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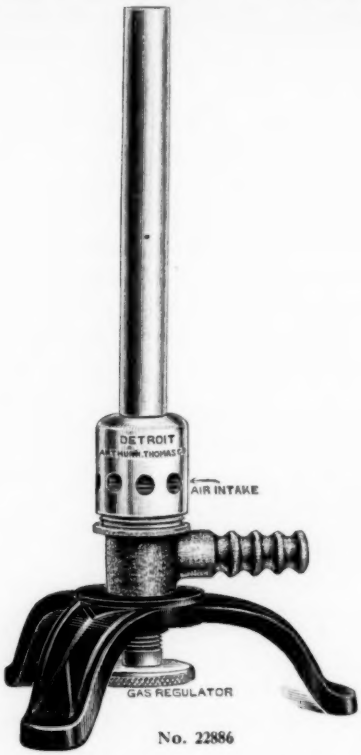

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THE EFFECT OF ACETONE AND OF β-HYDROXYLBUTYRIC AND ACETOACETIC ACIDS ON THE BLOOD CATALASE.

By W. E. BURGE.

(From the Physiological Laboratory, University of Illinois, Urbana.)

(Received for publication, January 10, 1919.)

Of the great number of investigators (1) who have worked on the respiratory metabolism in diabetes some have found a decrease in metabolism, some an increase, while others have found no change from the normal. Benedict and Joslin (2) have carried out the most extensive investigations of all those who have worked on diabetes in man and found an increase of about 15 per cent in metabolism. Benedict suggests that this increase in oxidation may be due to the stimulation of the cells of the body by the accumulation of substances such as β-hydroxylbutyric and acetoacetic acids and acetone. From the literature it would seem that the preponderance of evidence is in favor of those who claim that oxidation is increased in diabetes.

We had already found that when oxidation was increased, as for example after the ingestion of food, there resulted an increase in catalase due to the stimulation of its production by the alimentary glands, particularly the liver, and that when oxidation was decreased, as in chloroform narcosis, there resulted a corresponding decrease in catalase due to the direct destruction and decreased output of this enzyme from the liver. These and similar observations were taken to mean that catalase is the enzyme in the body principally responsible for oxidation. The present investigation was begun in an attempt to determine whether the introduction into animals of acetone and β-hydroxylbutyric and acetoacetic acids, substances which are found in large quantities in severe cases of diabetes, would stimulate the alimentary glands, particularly the liver, to an increased output of catalase, and thus produce an increase in oxidation. The animals used were rab-

bits and dogs. The β-hydroxylbutyric and acetoacetic acids were given in the form of sodium salts. The amounts of the substances will be given in the description of the individual experiments. The catalase was determined by adding 0.5 cc. of blood to diluted hydrogen peroxide at approximately 22°C. in a bottle, and the amount of gas liberated in 10 minutes was taken as a measure of the amount of catalase in the 0.5 cc. of blood.

After exposing the jugular vein and opening the abdominal wall with the use of a local anesthetic, quinine and urea hydrochloride, 5 gm. per kilo of 30 per cent solutions of acetone and β-hydroxylbutyric and acetoacetic acids were introduced into the upper part of the small intestines of rabbits. Determinations of the catalase of blood taken from the jugular were made before as well as at 15 minute intervals after the introduction of the materials. The results of the determinations are given in Fig. 1. The ordinates represent amounts of catalase measured in cc. of oxygen, and the abscissæ, time in minutes. It may be seen under acetone that previous to the introduction of this substance into the intestine, 0.5 cc. of blood liberated 285 cc. of oxygen from hydrogen peroxide; 30 minutes after the introduction, 0.5 cc. of blood liberated 350 cc. of oxygen, and after 45 and 60 minutes, it liberated 360 cc. of oxygen. It may be seen also under acetoacetic acid and β-hydroxylbutyric acid that the introduction of these substances into the intestine produced an increase in catalase.

The object of the second part of this paper was to determine the mode of action of acetone and β-hydroxylbutyric and acetoacetic acids in producing an increase in catalase. We had already observed that the catalase content of the blood of the liver was always 15 to 20 per cent higher than that of the blood from any other part of the body. This observation was interpreted to mean that the liver was continually replenishing the blood and hence the tissues with catalase. We had also found that after the ingestion of food the catalase of the blood of the liver was increased more rapidly than that from any other part of the body. This observation was taken to mean that the absorbed food materials, being carried to the liver, were stimulating this organ to an increased output of catalase. It was also found that when the liver was cut out of the circulation by means of an Eck fistula and by tying off the hepatic arteries, the introduction

of ethyl alcohol into the alimentary tract produced a very small or no increase, whereas normally this substance produces a large increase in catalase. From these observations, the conclusion was drawn that the liver is the principal organ in which catalase is formed and given off to the blood. Stated more specifically,

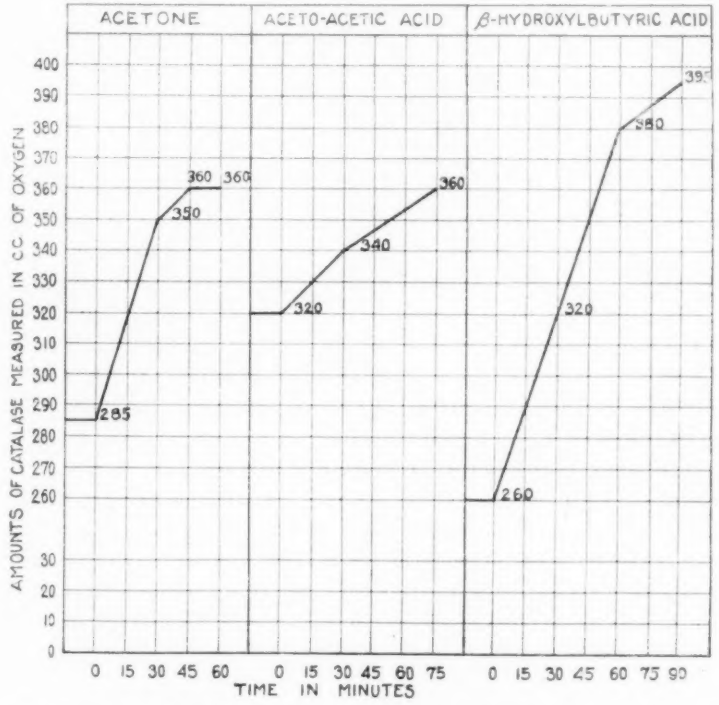

FIG. 1. Curves showing the increase produced in the catalase of blood of rabbits by the introduction into the intestine of acetone, β-hydroxylbutyric acid, and acetoacetic acid.

the second part of this paper is concerned with determining whether the increase in catalase after the introduction of acetone and β-hydroxylbutyric and acetoacetic acids is due to the stimulation of the liver to an increased output of this enzyme.

After etherizing dogs and opening the abdominal wall, 5 gm. per kilo of 30 per cent solution of acetone, β-hydroxylbutyric acid,

and acetoacetic acid were introduced into the upper part of the intestine. The catalase in 0.5 cc. of blood taken directly from the liver and the portal and jugular veins was determined before as well as at 15 minute intervals after the introduction of the

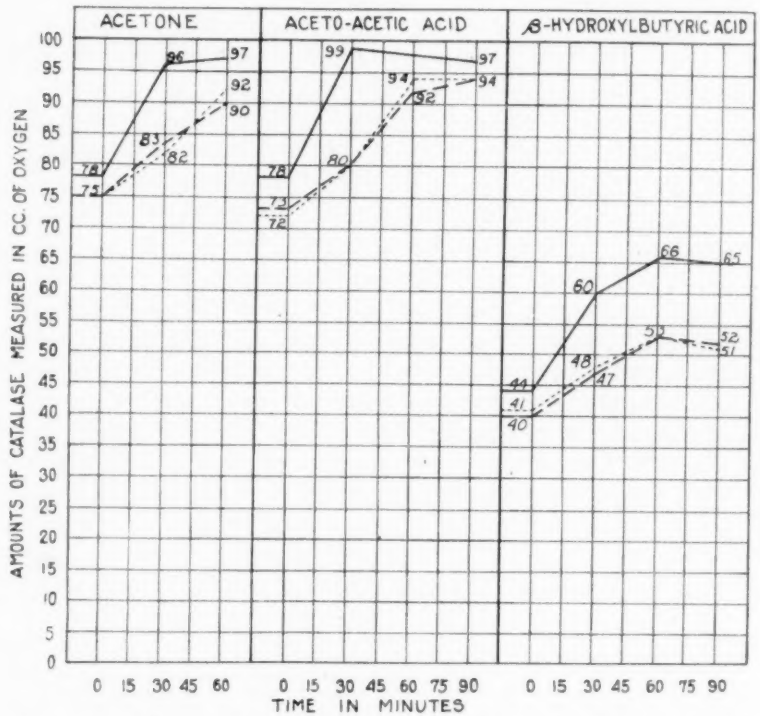

FIG. 2. Curves showing the increase in the catalase of the blood of the liver and portal and jugular veins produced by the introduction of acetone and β-hydroxylbutyric and aceto-acetic acids into the intestine of dogs. The solid line curves show amount of catalase in the blood of the liver, broken line curves the amount in the portal vein, and the dotted line curves the amount in the blood of the jugular vein.

different materials. The blood of the liver was collected from a superficial incision made in this organ. The results of the determinations are given in Fig. 2. The ordinates represent amounts of catalase measured in cc. of oxygen, and the abscissæ, time in minutes. The solid line curves were constructed from data ob-

tained from the blood of the liver, the broken line curves from the blood of the portal vein, and the dotted line curves from the blood of the jugular vein. It may be seen under acetone that previous to the introduction of this material into the intestine, 0.5 cc. of blood from the liver liberated 78 cc. of oxygen from hydrogen peroxide, while 0.5 cc. of blood from the portal and jugular veins liberated 75 cc. 30 minutes after the introduction of the acetone, the catalase of the blood of the liver and the portal and jugular veins was increased as indicated by the increase in the amount of oxygen liberated from hydrogen peroxide. The catalase of the blood of the liver was increased particularly during the first 15 minutes to a greater extent than that of the portal or jugular veins. This is taken to mean that the acetone was stimulating the liver to an increased output of catalase. It may be seen also that the introduction into the intestine of the acetoacetic and β-hydroxylbutyric acids produced an increase in catalase.

SUMMARY.

The increased oxidation in diabetes is attributed to the increase in catalase which is due to the stimulation of the liver to an increased output of this enzyme by acetone and β-hydroxylbutyric and acetoacetic acids.

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2. Benedict, F. G., and Joslin, E. P., *Carnegie Institution of Washington. Publication 136*, 1910; *Publication 176*, 1912.

LACTOSE, FAT, AND PROTEIN IN MILK OF VARIOUS ANIMALS.

By OTTO FOLIN, W. DENIS, and A. S. MINOT.

(From the Biochemical Laboratories of the Harvard Medical School and of the Massachusetts General Hospital, Boston.)

(Received for publication, January 20, 1919.)

During the past year we published descriptions of two methods[1] for the determination of lactose in milk, by means of which we were able to determine the sugar in milk rapidly, and with a considerable degree of accuracy. These methods have also the advantage that they may be carried out on relatively small quantities of material, a factor of importance when the milk of small animals is to be studied. In Tables I to III we have collected the results obtained by the use of our method in the examination of the milk of various animals. For the sake of completeness we have also included the figures for fat and protein.

Fat was determined by the Babcock method for the larger animals, while in the guinea pig, the rabbits, the cats, and the dog we have employed Bloor's nephelometric method[2] which requires but 1 cc. of milk for duplicate determinations. Protein was calculated from the nitrogen figures obtained by the Kjeldahl-Gunning method.

The figure given for cow's milk is an average of the analysis of the milk of twenty-one cows from the Hospital dairy; these cows included eleven Holstein, four Jersey, and six of mixed breed.

We are indebted to Dr. Fritz Talbot for the samples of milk of the goat, sheep, dog, mare, and women.

The data obtained on the cats and rabbits cover practically the entire period of active lactation, as we discontinued milking these animals only when the amount of milk obtainable was so small as to be inadequate for analysis. Our cats were fed on lean beef and whole milk, the rabbits on oats and cabbage.

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1918, xxxiii, 521.

[2] Bloor, W. R., *J. Am. Chem. Soc.*, 1914, xxxvi, 1500.

TABLE I.

Analyses of Rabbit's Milk.

Date of milking.	Lactose.	Fat.	Protein.	Remarks.
1918	*per cent*	*per cent*	*per cent*	
Mar. 23	1.45	8.7	13.75	Rabbit 1. Delivered Mar. 12.
" 25	2.00	8.4	14.3	
" 29	2.00	6.3	10.1	
Apr. 1	1.60	15.3	11.8	
" 10	0.90	33.0	13.12	Very little milk. Young rabbit practically weaned.
Apr. 9	1.00	18.33	11.37	Rabbit 4. Delivered 1.00 p.m., Apr. 9. First milk taken 4.00 p.m. of same day.
" 10	1.60	17.60	11.80	
" 12	2.60	14.40	11.37	
" 16	2.40	14.0	13.12	
" 20	1.90	13.6	13.57	
" 25	2.00	16.0	11.37	
May 1	1.90	13.2	12.23	
Apr. 18	1.1	44.4	21.87	Rabbit 5. Delivered Apr. 18, a.m. First milk obtained about 4 hrs. later.
" 20	1.8	13.4	14.00	
" 25	1.9		15.75	
May 1	1.8	16.6	11.8	

TABLE II.

Analyses of Cat's Milk.

Date of milking.	Lactose.	Fat.	Protein.	Remarks.
1918	*per cent*	*per cent*	*per cent*	
Mar. 25	2.3			Cat 1. Five kittens born Mar. 24, p.m.
" 26	2.5	10.5	6.5	
Apr. 2	4.0	10.6	9.6	
" 10	3.8	10.6	10.9	
" 12	3.8	10.5	15.3	
" 16	3.4	17.3	11.7	
" 20	3.9	8.2	10.5	
May 31	4.0	9.2	10.9	
Apr. 11	2.4	25.5	10.9	Cat 2. Three kittens born during night of Apr. 10–11.
" 12	2.3	10.3	15.3	
" 16	3.9		13.6	
" 20	3.9	10.0	8.3	
" 25	3.8	7.0	6.5	
May 1	3.8	6.2	10.5	
Apr. 9	3.0	18.1	7.87	Cat 3. Six kittens born 5 p.m., Mar. 25.
" 12	3.0	14.3	9.62	
" 16	2.9	12.3	14.00	
" 20	3.9	8.3	9.62	
" 23	3.8	8.6	10.00	

TABLE III.

Analyses of the Milk of Various Animals.

Animal.	Lactose.	Fat.	Protein.	Remarks.
	per cent	*per cent*	*per cent*	
Guinea pig.	3.0	8.3	8.75	Milk taken 24 hrs. after delivery.
Pig.	4.0	3.68	7.93	" " 5 wks. " "
Goat 1.	4.7	2.4	2.14	Pure Saanen (Swiss) goat. Milk taken 8 wks. after kidding.
" 2.	4.9	5.4	2.44	Grade $\frac{3}{8}$ Anglo-Nubian goat. Milk taken $4\frac{1}{2}$ mos. after kidding.
" 3.	5.46	4.2	3.32	Grade Anglo-Nubian goat, 50 per cent pure. Milk taken $2\frac{3}{4}$ mos. after kidding.
" 4.	5.2	3.7	3.72	Common American goat. Milk taken 3 mos. after kidding.
Sheep.	5.4	2.6	5.00	Hampshire. 4 yrs. old. Milk taken $2\frac{1}{2}$ mos. after delivery.
Dog.	2.6	10.0	10.60	Russian wolf hound. Pups 3 days old.
Mare 1.	8.5	0.9	2.40	
" 2.	7.7	1.0	2.70	Before being nursed by foal.
" 2.	8.0	1.6	2.70	After " " " "
" 3.	7.4	0.1	1.72	Average of results on 3 samples. Foal $3\frac{1}{2}$ mos. old.
Rabbit.	1.8	12.1	11.4	Average of results on 19 samples of milk from 6 rabbits.
Cat.	3.4	10.9	11.1	Average of results of 26 samples of milk from 4 cats.
Cow.	4.54			Average of results on milk from 21 cows.
Woman.	7.06		1.39	Average of results on milk from 87 normal women.

METHODS FOR THE QUANTITATIVE DETERMINATION OF THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF MILK.

By W. DENIS and A. S. MINOT.

(From the Chemical Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, January 25, 1919.)

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About a year ago work was commenced on a study of the non-protein fraction of milk. When this investigation was planned it was believed that the methods now in use for the determination of the non-protein constituents of blood could be used with advantage for the analysis of milk. Preliminary experiments soon proved, however, that our supposition was incorrect. The relatively large amounts of lactose and of fat present in milk, and the difference in the chemical properties of the proteins of milk as compared with those of blood, have made it necessary for us to spend a considerable amount of time on working out analytical methods to fit our needs. The methods described below have been used by us in the examination of a considerable number of samples of cow's and human milk and with this material have given good results.

Total Non-Protein Nitrogen.

A necessary preliminary to the determination of non-protein nitrogen in milk is a procedure for removing not only the protein, but also lactose and fat. The necessity of complete removal of protein is obvious, and, while it might be possible to devise some

method of oxidation which would cope successfully with the problem of oxidizing the large quantities of fat and sugar present in milk, the easiest solution of the question lies in preliminary removal of these substances.

Several procedures have been proposed for this determination. The older investigators use alcohol as a protein precipitant. Ritthausen[1] and later Munk[2] have recommended cupric hydroxide, Siebelein[3] has made extensive use of tannic acid, while Simon[4] has obtained good results with *m*-phosphoric and trichloroacetic acids.

In the procedure described below the desired result is obtained by two precipitations; in the first protein and fat are removed by means of copper sulfate and heat, while in the second the lactose is disposed of by means of cupric hydroxide. We have found that the method of Salkowski[5] for the precipitation of glucose by alkaline copper solutions may also be applied to lactose, but have found the use of calcium hydroxide as recommended by Van Slyke[6] superior for our purpose to the sodium hydroxide used by Salkowski, as the excess of calcium hydroxide remaining in solution may be easily and quantitatively precipitated as calcium oxalate. Ammonium salts, urea, creatine, and creatinine may be carried through the above precipitations without loss. Uric acid is completely precipitated so that our results do not include the nitrogen of this substance. As the uric acid nitrogen of milk is but a small fraction of the total non-protein nitrogen, the error due to its loss is small. For some time we found it impossible to effect even an approximately complete recovery of amino-acids; in some cases as much as 65 per cent of the amino nitrogen added (in the form of alanine or leucine) was lost during the process. The property possessed by amino-acids to precipitate in the form of insoluble copper compounds, when these bodies are treated with alkaline cupric hydroxide, has long been known and has of recent years been made use of by Kober and

[1] Ritthausen, H., *J. prakt. Chem.*, 1877, xv, 329.
[2] Munk, I., *Virchows Arch. path. Anat.*, 1893, cxxxiv, 501.
[3] Siebelein, J., *Z. physiol. Chem.*, 1889, xiii, 157.
[4] Simon, G., *Z. physiol. Chem.*, 1901, xxxiii, 466.
[5] Salkowski, E., *Z. physiol. Chem.*, 1879, iii, 79.
[6] Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxii, 455.

Sugiura[7] for the quantitative determination of amino nitrogen. We have found it possible to prevent the formation of these insoluble amino-copper compounds, and incidentally to obtain complete recovery of amino-acids carried through the three precipitations, by the addition of a small amount of ammonia-free formaldehyde, after the first precipitation and before the addition of lime.

The method as finally worked out for use with cow's milk is as follows: 10 cc. of milk are pipetted into a 100 cc. volumetric flask, and to this are added approximately 50 cc. of water and 20 cc. of 10 per cent copper sulfate solution to which has been added a sufficient amount of sulfuric acid to make a 0.005 N solution. The flask is shaken and placed in a boiling water bath for 20 minutes. It is then removed from the water bath, cooled, made up to volume with distilled water, and the contents are filtered through a dry filter paper. 75 cc. of this filtrate are transferred to a 100 cc. volumetric flask and treated with 1 cc. of 30 per cent formaldehyde and 20 cc. of a 10 per cent suspension of calcium oxide, and after standing at room temperature for 30 minutes the solution is made up to volume and filtered through a dry filter paper. The prescribed time given for the precipitation should not be curtailed, as, if this is done, complete precipitation of lactose will not be secured. If, on the other hand, the time of standing is unduly prolonged to an hour or more, decomposition of the formaldehyde begins with slow evolution of gas and consequent foaming and loss of material.

The filtrate should be clear and colorless, free from lactose, and practically free from all but minute traces of copper. This filtrate is then treated with 0.80 gm. of a mixture consisting of five parts of powdered oxalic acid and two parts of powdered potassium oxalate and centrifuged to remove the precipitated calcium oxalate.

While the amount of precipitant recommended removes in the great majority of cases all the calcium, it is well, after the tubes are removed from the centrifuge, to add a small crystal of oxalate to make sure that the precipitation is complete. 20 cc. of this filtrate (which is equivalent to 1.5 cc. of milk) are pipetted into a

[7] Kober, A., and Sugiura, K., *J. Am. Chem. Soc.*, 1913, xxxv, 1546.

20 by 200 mm. pyrex glass test-tube; a glass bead or fragment of quartz, and 1 cc. of a digestion mixture prepared by mixing 100 cc. of 85 per cent phosphoric acid, 300 cc. of concentrated sulfuric acid, and 15 cc. of 10 per cent copper sulfate solution are added, and the mixture is evaporated almost to dryness either with a direct flame or in a bath of saturated calcium chloride solution. When practically all the water has been driven off, the tube is heated by means of a microburner for 1 minute after the contents become colorless. During the final period of heating the mouth of the tube should be kept covered by means of a watch-glass or an inverted 25 cc. Erlenmeyer flask. The total time of heating after the evaporation has been completed should not consume more than 3 minutes. As soon as the tube and its contents have cooled, water is added and the ammonia determined either colorimetrically by direct Nesslerization according to the method for the determination of non-protein nitrogen in blood described by Folin and Denis,[8] or by titration with 0.02 N sodium hydroxide and methyl red after aeration according to the method of Folin and Farmer,[9] or distillation according to the procedure of Bock and Benedict.[10] In practically all of our work we have used the colorimetric method, but we have had sufficient experience with the other procedures to know that good results are obtainable.

While the procedure outlined above gives excellent results with cow's milk, it requires considerable modification as regards the first precipitation before it can be used satisfactorily with human milk. When human milk is to be used, the precipitation of protein and fat is conducted as follows:

To 10 cc. of milk contained in a 100 cc. volumetric flask are added 20 cc. of 10 per cent copper sulfate solution (which should not contain added sulfuric acid as called for in the directions for use with cow's milk), about 30 to 50 cc. of water, and 1.5 cc. of 10 per cent disodium phosphate solution. The flask is heated for 20 to 30 minutes in a boiling water bath, then cooled, made up to volume with distilled water, and filtered through a dry filter paper. The remainder of the process is carried out as described for cow's milk.

[8] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.
[9] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.
[10] Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 47.

In Table I are given a few of the results obtained when known amounts of pure solutions of some of the constituents of the non-protein fraction of milk were carried through the process outlined above.

TABLE I.

Results on Recovery of Several Constituents of Non-Protein Fraction of Milk.

Substance added.	Quantity added.	Quantity recovered.
	mg.	*mg.*
Urea	2.00	1.90
"	3.00	2.93
"	5.00	5.10
Alanine N	0.50	0.54
" "	1.00	0.98
" "	2.00	1.98
Leucine N	0.50	0.50
" "	2.00	2.00
Creatinine	0.15	0.16
"	1.00	0.98
Creatine	0.50	0.50
"	2.00	1.97
Uric acid	0.20	0
" "	1.00	0
" "	2.00	0

Urea.

Urea may be determined by Marshall's urease[11] method which is well suited for use with milk. We have found it more convenient for our purpose to determine the ammonia produced from the urea colorimetrically instead of by titration as recommended by Marshall. The procedure as used by us contains no new features and the description given below is inserted here merely for completeness.

3 cc. of human, or 5 cc. of cow's milk, are allowed to stand for 30 minutes in contact with 2 cc. of an extract of Jack bean. 1 gm. of solid potassium carbonate and one to two drops of kerosine are then added, and the mixture is aerated for 15 minutes. The ammonia is collected in a 100 cc. volumetric flask containing 2 cc. of 0.1 N hydrochloric acid and 25 cc. of water. At the expiration

[11] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.

of the period of aeration the contents of the flask are made up with distilled water to a volume of 60 to 70 cc. and there are added 15 cc. of Nessler's solution; distilled water is then added to the mark and the solution read in a colorimeter against a suitable standard. For normal milk, the most convenient standard is one containing 0.5 mg. of ammonia nitrogen in a volume of 100 cc.

Creatinine and Creatine.

The only quantitative method for the determination of creatinine and creatine in milk is that of Folin.[12] This method (as regards its application to blood) has recently received severe criticism from several sources.[13] When an attempt was made to use this procedure it was found that the strictures attached to the method for blood analysis also apply in the case of milk examination; in fact, the rapid increase in color noted when blood filtrates are used, an increase which, as has been pointed out, is not in proportion to changes taking place in the creatinine standard, is even more pronounced in milk than in blood.

Recourse was therefore had to a procedure which has in our hands given good results with blood.[14] This modification consists in the use of *m*-phosphoric acid as a protein precipitant instead of picric acid as recommended in the original Folin method. While this *m*-phosphoric acid filtrate gave good results with blood, it was found that in the case of milk the results were no better than those obtained by the picric acid precipitation. It was possible to prove experimentally that the rapid increase in color, obtained when alkali and picric acid are added to deproteinized milk filtrates (whether the protein precipitant consisted of *m*-phosphoric, trichloroacetic, or picric acid) is largely if not entirely due to the relatively high lactose content of these filtrates; the problem of securing a method for the relatively accurate determination of creatinine and creatine in milk may therefore be solved in one of two ways. In the first we have added to the

[12] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[13] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413. Hunter, A., and Campbell, W. R., *ibid.*, 1917, xxxii, 195. Greenwald, I., and McGuire, G., *ibid.*, 1918, xxxiv, 103.

[14] Denis, W., *J. Biol. Chem.*, 1918, xxxv, 513.

standard creatinine solution an amount of lactose equivalent to that contained in the filtrate, as a result of which unknown and standard darken at the same rate of speed. The procedure is as follows:

10 cc. of milk contained in a 50 cc. volumetric flask are treated with an equal volume of water and 3 cc. of a freshly prepared 20 per cent solution of *m*-phosphoric acid, and after standing for an hour the flask is filled to the mark with distilled water and the contents are mixed and poured on a dry filter. For the determination of preformed creatinine, 10 cc. of this filtrate are transferred to a 25 cc. volumetric flask containing 10 cc. of picric acid solution (1.2 per cent) and 1.5 cc. of 10 per cent sodium hydroxide. After standing at room temperature for 10 minutes the solution is diluted to volume, placed in the cup of a colorimeter, and read against a standard which has been standing for the same length of time. For normal cow's milk this standard consists of 10 cc. of a 0.9 per cent solution of lactose, 0.025 mg. of creatinine, 10 cc. of picric acid solution, and 1.0 cc. of 10 per cent sodium hydroxide made up to a final volume of 25 cc. For determinations on human milk a 1.4 per cent solution of lactose is substituted for the 0.9 per cent solution used in the case of cow's milk.

For the determination of total creatinine (creatine + creatinine) 10 cc. of the *m*-phosphoric acid filtrate are autoclaved at 120°C. for 30 minutes. After cooling, the solution is treated with picric acid and sodium hydroxide as described above, and after standing for 10 minutes is diluted to a volume of 50 cc. The standard for this determination contains 0.05 mg. of creatinine in a final volume of 50 cc. Lactose solution, etc., is added as described above. By the use of this method on a considerable number of samples of milk, both cow's and human, we obtained values for preformed creatinine of 1.1 to 1.4 mg. of creatinine per 100 cc. of milk, and of total creatinine, 3.1 to 4.0 mg. per 100 cc. of milk, values approximately half as great as those obtained by the Folin method. We have therefore attempted to check our results obtained by the above procedure by resorting to an entirely different method for getting rid of the interfering substances. To this end we have sought to obtain a method whereby the lactose and other active substances might be

removed from the reaction without at the same time destroying any creatine or creatinine. We have found such a precipitant in Salkowski's alkaline cupric hydroxide which precipitates lactose, fat, and protein without carrying down any creatine or creatinine.

The precipitation is best accomplished by the use of copper sulfate and calcium hydroxide as recently suggested by Van Slyke. The procedure is as follows: To 10 cc. of milk contained in a 50 cc. volumetric flask add 5 cc. of a 20 per cent solution of copper sulfate and 15 cc. of a 10 per cent suspension of calcium oxide. Add water to the mark, mix, and allow to stand for 30 minutes; centrifuge, and filter the supernatant liquid through a small dry filter paper. The filtrate should be absolutely clear and colorless and free from lactose and all but minute traces of protein.

To determine preformed creatinine transfer 10 cc. to a 25 cc. volumetric flask, add two drops of normal hydrochloric acid, 10 cc. of saturated picric acid, and 1 cc. of 10 per cent sodium hydroxide solution; let stand 10 minutes, then make up to the mark and read against a suitable standard. For normal milk a standard containing 0.025 mg. of creatinine in 10 cc. of distilled water, 10 cc. of picric acid, and 1 cc. of 10 per cent sodium hydroxide made up to 25 cc. volume is most convenient. For pathological material more concentrated standards are sometimes necessary. For the determination of total creatinine 10 cc. of filtrate are placed in a 50 cc. volumetric flask, treated with two drops of normal hydrochloric acid and 10 cc. of picric acid, and autoclaved for 30 minutes at 120°C. When cool, the mixture is treated with 1 cc. of 10 per cent sodium hydroxide and after standing for 30 minutes is made up to volume and read in a colorimeter against a suitable standard. For normal milk, the most convenient standard is one made to contain 0.05 mg. of creatinine, 10 cc. of picric acid, and 1 cc. of 10 per cent sodium hydroxide in 50 cc.

In the above determinations the addition of sodium hydroxide causes a precipitation of calcium hydroxide which must be centrifuged off before an attempt is made to use the solution in the colorimeter.

The values obtained on normal milk by this method are essentially the same as those obtained when the lactose standard is used and, as we believe it to be theoretically more sound in principle than the former procedure, we have in the latter part of this work adopted it exclusively.

Amino Nitrogen.

The amino nitrogen may be determined by means of Van Slyke's[15] nitrous acid method. For work with milk the micro-apparatus should be used. At first an attempt was made to use 95 per cent ethyl alcohol as a protein precipitant as recommended by Van Slyke and Meyer[16] in their work on blood. While with sufficient care the Van Slyke-Meyer technique was found to give quantitative results in recovering amino-acids added to milk, a short trial convinced us that it was not a practical method for use with this fluid, as so much of the fat present in milk is extracted by the alcohol and later deposited in the deaminizing bulb, and to some extent in the burette, that it was found difficult, if not impossible, to make accurate readings of the latter; an added disadvantage is the fact that the presence of such large quantities of fat makes it necessary to clean the instrument thoroughly after each determination.

Precipitation of the protein by acetic acid followed by trichloroacetic acid as recommended for blood by Bock[17] was also attempted, but was found to be so time-consuming on account of slow filtration that it was abandoned.

After a trial of various protein precipitants we have decided on the use of copper acetate and acetic acid as being most satisfactory for this determination, since by this means it is possible to obtain clear, rapidly filtering filtrates without loss of amino nitrogen. The method as finally worked out for cow's milk is as follows:

20 cc. of milk are pipetted into a 200 cc. volumetric flask, and there are then added 40 cc. of 0.01 N acetic acid, 10 cc. of 5 per cent copper acetate, and 50 to 60 cc. of distilled water. The flask is placed in a boillng water bath for 20 to 30 minutes and at the end of this period of heating, 1 cc. of 15 per cent potassium oxalate solution is added and the mixture cooled, made to volume, and filtered through a dry filter paper. To the filtrate there are now added 0.5 gm. of powdered potassium oxalate, and after shaking the flask for 1 or 2 minutes the precipitate may be re-

[15] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.
[16] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.
[17] Bock, J. C., *J. Biol. Chem.*, 1916-17, xxviii, 357.

moved by centrifuging, leaving a perfectly clear supernatant liquid.

The precipitate may, if desired, be removed by filtration instead of by centrifugation, but in this case the liquid must be allowed to stand for 1 hour as otherwise it is difficult, if not impossible to obtain a clear filtrate.

The addition of potassium oxalate is made in order to remove calcium salts, and while the two filtrations as outlined above may seem unnecessary and cumbersome, it has been found impossible to obtain a filtrate which would remain clear on boiling when all the oxalate was added in the first precipitation; while if the second addition of oxalate is omitted so much solid material precipitates out when the filtrate is evaporated to small volume in preparation for the amino nitrogen determination that it is practically impossible to effect a quantitative transfer to the deaminizing bulb.

For the amino nitrogen determination 50 cc. of the final filtrate are evaporated to a volume of 1 to 2 cc. and the determinations are carried out by means of Van Slyke's micro-apparatus.

For human milk the method of precipitation described above gives poor results, so that with this fluid the following method should be used.

20 cc. of breast milk contained in a 200 cc. volumetric flask are treated with 10 cc. of 5 per cent copper acetate solution, 1 cc. of a 10 per cent solution of disodium phosphate, and 60 to 80 cc. of distilled water. The mixture is heated in a boiling water bath for 20 to 30 minutes, then cooled, made up to volume with distilled water, and the contents are mixed and poured on a dry filter. 50 cc. of the filtrate are evaporated to a volume of about 2 cc. and used for the determination of amino nitrogen. The addition of two to three drops of glacial acetic acid facilitates transfer to the deaminizing bulb.

The advisability of evaporating solutions of amino-acids in open vessels in the presence of relatively large quantities of copper and lactose may well be questioned. The following results indicate, however, that under the experimental conditions described above no destruction of amino nitrogen can be demonstrated during this procedure. These results were obtained on an amino-acid mixture prepared by hydrolyzing Kahlbaum's pure casein with

20 per cent sulfuric acid for 40 hours. The greater part of the sulfuric acid was then precipitated by means of barium hydroxide and the solution finally brought to a faintly acid reaction by means of sodium hydroxide. Amino nitrogen determinations were then made on 1 cc. of the solution (*a*) without the addition of any other substance, (*b*) with the addition of 0.2 gm. of copper sulfate to 1 cc. of the solution, (*c*) with the addition of 0.2 gm. of copper sulfate and 0.1 gm. of lactose to 1 cc. of the solution. In (*b*) and (*c*) the mixture was made up to a volume of approximately 50 cc. and evaporated on the sand bath to a volume of 1 to 3 cc. under exactly the same experimental conditions as were used for the determination of amino nitrogen in milk. The results are given in Table II.

TABLE II.

Amino N per 100 cc. of solution before treatment.	Amino N per 100 cc. of solution after heating with $CuSO_4$.	Amino N per 100 cc. of solution after heating with $CuSO_4$ and lactose.
mg.	*mg.*	*mg.*
30.34	31.48	32.00
31.48	31.48	32.00
31.48	32.00	
32.00		

TABLE III.

Comparisons of Results Obtained on Cow's Milk by Alcohol and by Copper Precipitations.

No. of milk.	Amino N per 100 cc. milk.	
	Alcohol precipitation.	Copper precipitation.
	mg.	*mg.*
4	4.86	4.86
3	4.52	4.62
10	5.74	5.78

In Table III are presented à few results in which amino nitrogen was determined in cow's milk after alcohol precipitation by the technique of Van Slyke and Meyer and by the copper precipitation described above. The results obtained by the two methods are almost identical.

Uric Acid.

Uric acid determinations in blood have almost invariably been made with Salkowski's ammoniacal silver-magnesium reagent, preceded by precipitation with acetic acid and evaporation to small volume. Such a procedure is absolutely unsuited for use with milk on account of the large quantities of lactose present, which, when the milk filtrates are evaporated to small volume, leaves a sticky mass producing strong reduction of the ammoniacal silver reagent.

For our purpose the method of precipitation of uric acid with alkaline zinc acetate solution recommended by Morris[18] has proved excellent, as by this method it is possible to remove quantitatively small amounts of uric acid from relatively large volumes of lactose solution. Recently Curtman and Lehrman[19] have suggested the use of nickel acetate in place of zinc acetate as recommended by Morris. In a trial of nickel as a uric acid precipitant we have obtained excellent results, but as we have been unable to find that for our purpose this reagent possesses any advantage over zinc acetate we have continued our use of the latter.

The method as finally worked out is as follows: 20 cc. of milk contained in a 100 cc. volumetric flask are treated with 40 cc. of 0.01 N acetic acid and 1 cc. of 10 per cent zinc acetate solution; the mixture is heated in a boiling water bath for 15 to 20 minutes and is then cooled, made up to volume, and filtered through a dry filter paper. The filtration should be rapid and the filtrate absolutely clear. 75 cc. of filtrate are treated with 3 cc. of a 10 per cent solution of zinc acetate and 4 cc. of 20 per cent sodium carbonate solution, allowed to stand at room temperature for 10 minutes, and then centrifuged. The gelatinous precipitate of zinc carbonate settles rapidly and completely, is washed once with cold water, and the tubes are then centrifuged a second time. After pouring off the wash water as completely as possible the residue is treated with 2 cc. of 50 per cent acetic acid, then transferred by means of about 25 to 35 cc. of water to an Erlenmeyer flask; the contents of the flask are heated to boiling, and the

[18] Morris, J. L., *J. Biol. Chem.*, 1916, xxv, 205.

[19] Curtman, L. J., and Lehrman, A., *J. Biol. Chem.*, 1918, xxxvi, 157.

zinc is precipitated with hydrogen sulfide. When precipitation is complete, the liquid is filtered, the zinc sulfide precipitate washed twice with hot water, the filtrate and washings are boiled rapidly until entirely free from hydrogen sulfide, and the volume is reduced to approximately 15 cc. After cooling, 1 cc. of phosphotungstic acid reagent[20] and 10 cc. of 20 per cent sodium carbonate solution are added and the volume is made up to 25 or 50 cc. ac-

TABLE IV.

Results on Recovery of Non-Protein Bodies Added to Cow's Milk.

Substance added.	Mg. per 100 cc. milk.			
	Amount present.	Amount added.	Amount recovered.	Theory.
Urea N	10.0	5	14.8	15.0
" "	10.0	10	20.2	20.0
" "	10.0	10	19.8	20.0
Amino N	7.02	2	8.98	9.02
" "	7.02	4	11.42	11.02
" "	7.02	6	12.91	13.02
Creatinine	1.1	1	2.0	2.1
"	1.1	2	3.0	3.1
Creatine	2.2	1	3.1	3.2
"	2.2	2	4.1	3.9
Uric acid	1.5	1	2.2	2.5
" "	1.5	3	4.3	4.5
" "	1.5	5	6.4	6.5

TABLE V.

Non-Protein Nitrogenous Constituents of Milk.

Source of material.	Mg. per 100 cc. milk.					
	Total non-protein N.	Urea N.	Amino N.	Preformed creatinine.	Creatine.	Uric acid.
Cow, Dairy I	21.9	10.0	4.03	1.35	2.30	1.4
" " II	21.0	9.0	4.19	1.40	2.40	1.6
" " III	26.0	9.0	4.50	1.5	2.40	1.6
Mother I	26.0	12.0	4.58	1.1	3.9	2.2
" II	37.0	12.1	8.46	1.6	3.2	2.7
" III	36.0	13.8	8.58	1.8	3.4	2.7

[20] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.

cording to the degree of color. Readings are then made in a colorimeter against a suitable standard uric acid solution. The standard which is most generally useful is one containing 0.5 mg. of uric acid in a volume of 100 cc.

The following results indicate the completeness with which uric acid can be recovered by the above procedure from relatively large quantities of lactose solution:

Uric acid added.	7 per cent lactose solution.	Uric acid recovered.
mg.	*cc.*	*mg.*
0.2	10	0.19
1.0	50	1.0
1.0	100	0.97

In Table IV are presented the results obtained by adding known quantities of urea, amino-acids, creatine, creatinine, and uric acid to cow's milk.

Table V contains the results obtained on a few samples of cow's and human milk. These results are introduced to give an idea of the general level at which the non-protein nitrogenous bodies occur in these fluids.

The cow's milk was purchased in the open market and represents the product sold by three large milk distributers in Boston. The samples of human milk were from three normal women. For the latter specimens we are indebted to Dr. Fritz Talbot.

For some time we have been engaged by the help of the methods described above in the accumulation of data concerning the non-protein constituents of milk. The results of this work will be published shortly.

DETERMINATION OF THE DISTRIBUTION OF NITROGEN IN CERTAIN SEEDS.*

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(Received for publication, January 9, 1919.)

Recent progress in the chemistry of protein nutrition has demonstrated the importance of the rôle played by the amino-acids and sufficient evidence is at hand to show that protein nutrition is in reality amino-acid nutrition. Therefore for the purposes of human nutrition and of animal husbandry an exact knowledge of the amino-acid content of foodstuffs is essential. Methods of protein analysis which furnish accurate results expeditiously are therefore of the utmost importance. The Van Slyke method when applied to isolated and purified proteins partially meets this need but, obviously, the method would be of still greater value if it could be applied to the direct analysis of seeds without previous isolation of the proteins. In the investigation herein presented this was attempted with the hope of obtaining at least approximate results.

It is evident, however, that the possible sources of error in such an application of the Van Slyke method are many. Gortner and Blish[1] have shown that when tryptophane or a protein containing this amino-acid is hydrolyzed with hydrochloric acid in the presence of dextrose, the humin nitrogen is greatly increased. Grindley and Slater[2] regard the presence of carbohydrates to be a source of error in the hydrolysis of seed material for the purpose of determining its amino-acid content. They intimate, moreover,

* A brief notice of this work was published in *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 192.

[1] Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.

[2] Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.

that possibly the presence of glycerol or of glycerides may increase the humin nitrogen.

In the consideration of sources of error in the determination of the amino-acid content of seeds, it seems worth while to take into account the presence in the seeds of forms of nitrogen other than protein, such as amines and free amino-acids, and, important in this sort of analysis, the nucleic acid which is a part of the seed embryo. Information concerning the plant nucleic acids is limited. That of yeast and that of the wheat embryo have been thoroughly investigated by Osborne[3] and by Levene,[4] who believe that the two are practically identical, and it is probable that all the plant nucleic acids are closely related or have a very similar structure.

TABLE I.

Nitrogen Distribution in Nucleic Acid of Yeast as Determined by the Van Slyke Method.

Sample, 3 gm.	Total N.	Amide N.	Humin N with lime.	Humin N, bases.	Total N, bases.	Amino N, bases.	Arginine N.	Total N, filtrate.	Amino N, filtrate.
Gm.....	0.4752	0.1140	0.05166	0.1074	0.1324	0.024	0.0714	0.0602	0.017
Per cent of total (uncorrected).		24.00	10.87	22.09			15.03	12.66	

For the purpose of determining the behavior of plant nucleic acid in the Van Slyke analysis, yeast nucleic acid, which was free from material giving the biuret reaction and did not respond to qualitative tests for ammonium acetate, was hydrolyzed 25 hours with 20 per cent hydrochloric acid and subjected to the usual procedure.

Examination of the results given in Table I shows that a high percentage of humin nitrogen was precipitated with the bases, which may be explained by the fact that it is impossible to decompose completely the phosphotungstate precipitate of the bases with ether-amyl-alcohol mixture and dilute hydrochloric acid as recommended by Van Slyke.[5] Although yeast nucleic acid does

[3] Osborne, T. B., and Harris, I. F., *Z. physiol. Chem.*, 1902, xxxvi, 85.
[4] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1910, xliii, 3164.
[5] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

not contain arginine, the analysis indicates the presence of 15 per cent of arginine nitrogen. This is probably derived from the guanine, adenine, and cytosine of the nucleic acid, since these compounds are decomposed by boiling with concentrated caustic alkali as in the procedure used for the determination of arginine.

Table II gives the results of analyses of cottonseed flour, tomato seed flour, cow peas, jack beans, corn, corn germ, wheat, kafir, and kafirin, the alcohol-soluble protein of kafir.[6] With the exception of cottonseed flour and tomato seed flour, the seeds and grains were prepared for analysis by fine grinding in a suitable mill. They were used without further drying and without extracting the fats. The cottonseed flour was the well known commercial article. Tomato seed flour is the ground press cake of tomato seeds obtained as a by-product in the manufacture of tomato pulp. The seeds yield, on pressing, from 15 to 20 per cent of oil. Portions of the ground material were weighed, hydrolyzed with 20 per cent hydrochloric acid, and then treated exactly as recommended by Van Slyke.

By comparing the results given in Table II with those of others who have analyzed the same sort of material (Grindley and Slater,[2] Nollau,[7] and others), it will be noted that there is a widely varying agreement. The results of Nollau have been criticized by Grindley and Slater, who pointed out that Nollau's procedure was not comparable with theirs. Grindley and Slater followed the same procedure that was used in obtaining the results recorded in Table II, and these results agree fairly well with theirs. Differences noted may be due to the fact that different samples of seeds may vary in composition.

In the analysis of kafir corn and of tomato seed a deficiency in histidine nitrogen was found, calculations yielding minus quantities. The presence of hisitidine in tomato seed is yet in doubt. The solution of the hexone bases of kafir gives a positive reaction with bromine for this amino-acid, and histidine nitrogen, 1.64 per cent corrected, was found in the kafir prolamine. It seems justifiable then in the case of the whole kafir to assume that most of the small amount of histidine present remained in solution and,

[6] Johns, C. O., and Brewster, J. F., *J. Biol. Chem.*, 1916–17, xxviii, 59.
[7] Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.

TABLE II.

Distribution of Nitrogen in Certain Seeds as Determined by the Van Slyke Method.

	Weight of sample.	Total N.	Nitrogen in per cent of total nitrogen.								
			Amide N.	Humin N in lime.	Humin N precipitated with bases.	Arginine N.	Histidine N.	Cystine N.	Lysine N.	Amino N in filtrate from bases.	Non-amino N in filtrate from bases.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Cottonseed flour	4	0.3185	10.00	10.97		19.73	3.87	1.11	6.69	43.50	5.27
Tomato seed (pressed)	4	0.2622	10.38	12.03		16.32	None found.	1.27	8.39	45.86	5.09
Cow pea (*Vigna sinensis*)	8	0.3277	9.88	5.34	2.87	17.68	3.64	1.23	5.98	49.12	4.08
Jack bean (*Canavalia ensiformis*)	12	0.3969	7.56	6.35	4.11	9.83	6.08	0.79	9.90	45.54	9.40
Corn (*Zea mais*)	12	0.2220	13.94	9.35		7.75	2.46	1.60	2.06	57.86	6.28
Corn germ (pressed)	8	0.2648	12.69	14.71		11.04	5.84	None.	5.62	45.07	6.97
Wheat	12	0.3333	18.65	6.26	1.25	8.96	1.73	0.87	2.65	48.79	13.37
Kafir corn (*Andropogon sorghum*)	12	0.2808	16.34	4.85		7.17	1.32	1.02	1.68	59.01	7.69
Kafirin	3	0.4716	20.74	0.94	0.07	3.08	1.64	0.55	1.04	65.20	7.29

applying the correction for solubility of histidine, 1.32 per cent is recorded.

The figure for cystine nitrogen in kafirin recorded was also obtained by adopting the factor for solubility of the phosphotungstate. The barium sulfate precipitate, though unweighable, was present, and as the reagents used give no precipitate of barium sulfate in blank, it appears safe to adopt Van Slyke's correction of 0.0026 gm. as representing all the cystine nitrogen in solution.

CONCLUSIONS.

It is shown that when yeast nucleic acid is subjected to the Van Slyke procedure, 15 per cent of the total nitrogen of the acid appears in the arginine fraction, although the nucleic acid contains no arginine. This indicates that in the determination of the distribution of the nitrogen in materials containing nucleic acid by the method of Van Slyke, erroneous results may be obtained because some of the purine and pyrimidine nitrogen appears in the arginine fraction.

The nitrogen distribution in certain seeds and seed products was determined by the method of Van Slyke and the results agree in general with those obtained by others who have used the same method. Where there is lack of agreement this might be due to the fact that different samples of seed of the same species may vary in composition.

REDUCTION OF THE QUANTITY OF HUMIN NITROGEN FORMED IN THE HYDROLYSIS OF THE NITROGENOUS CONSTITUENTS OF FEEDINGSTUFFS.

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(Received for publication, January 28, 1919.)

In view of the importance which individual amino-acids had assumed in the biological evaluation of feedingstuffs, the junior author of this paper, with the assistance of Joseph, Slater, and Eckstein, attempted quantitatively to determine directly the combined amino-acids of feedingstuffs by the application of the Van Slyke method for the determination of the characteristic groups of amino-acids of proteins. In the publications[1] of the results of this work, the following facts have been clearly emphasized:

First, "that the Van Slyke method for the determination of the chemical groups characteristic of the amino acids [of proteins] can be applied directly to the quantitative determination of amino acids of feedingstuffs with at least a fair degree of accuracy."

Second, "that the object of the work . . . . is to determine the free and the combined amino acids . . . . of feedingstuffs, and not merely the amino acids and the amides resulting from the hydrolysis of the proteins of the feedingstuffs."

Third, "that the high results for humin nitrogen [due to the presence of the carbohydrates in feedingstuffs] constitute a source of error in the direct application of the Van Slyke method to the determination of the free and combined amino acids and amides of feedingstuffs."

Fourth, "that further study was being made to determine to what extent, if any, the so-called non-protein nitrogenous substances affect the quantitative determination of the amino acids of feedingstuffs."

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[1] Grindley, H. S., Joseph, W. E., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 1778. Grindley, H. S., and Slater, M. E., *ibid.*, 2762. Grindley, H. S., and Eckstein, H. C., *ibid.*, 1916, xxxviii, 1425. Grindley, H. S., *Proc. Am. Soc. Animal Production*, 1916, 133.

Fifth, "that the Van Slyke method for the determination of the chemical groups characteristic of the amino acids of proteins will prove of much value as applied to the quantitative determination of the free and combined amino acids . . . . of feedingstuffs when we become better acquainted with the details of the method, and succeed in adapting it to the specific conditions involved. At any rate, as yet, it is the only method that has been used for the determination of the amino acid content of feedingstuffs, and, since it undoubtedly gives approximately quantitative results, it should, until some better method is developed, be used in gaining knowledge, that will aid us in applying to the economic and nutritive valuation of the common feedingstuffs, the fast accumulating results as to the nutritive value of the amino acids."

Since our last publication the methods for this work have been decidedly improved. In the first place, the interference of some of the non-protein nitrogenous constituents such as the nitrogenous lipoids, the nitrogenous coloring matters, betaine, stachydrin, amygdalin, most of the alkaloids, and some of the amides has been obviated by completely extracting first with ether and then with cold absolute alcohol.

In the second place, a method has been perfected by which the percentages of the humin nitrogen formed in the hydrolysis of the nitrogenous constituents of feedingstuffs are reduced so that they compare favorably with those obtained for pure proteins. The essential features of this method are as follows: (1) extraction of the feedingstuffs with ether and then with cold absolute alcohol; (2) the conversion, as far as possible, of the insoluble carbohydrates into soluble carbohydrates by boiling the feedingstuffs with 0.1 per cent hydrochloric acid; (3) the separation of the greater part of the soluble proteins before hydrolysis from the soluble carbohydrates by neutralizing the mineral acid and precipitating with alcohol; (4) the hydrolysis of the small portion of the proteins remaining in solution with the soluble sugars by boiling with 5 per cent instead of 20 per cent hydrochloric acid for only a short time.

By these proceedings it is possible to separate the greater part of the carbohydrates from the main portion of the proteins before the latter are hydrolyzed and thus decidedly reduce the quantity of humin formed. Likewise since it is possible to hydrolyze the small amount of soluble proteins remaining in solution with the carbohydrates with dilute hydrochloric acid in a short time, a further reduction of the quantity of humin is obtained.

This method gives the following results for humin nitrogen expressed in per cent of the total nitrogen in the feedingstuff: corn 3.2, wheat 3.4, oats 4.5, and barley 3.9 per cent. The results for the humin nitrogen which were obtained in our earlier analysis of these feeds were as follows: corn 9.8, wheat 9.2, oats 9.9, and barley 8.8. The humin nitrogen content of some of the pure proteins as analyzed by Van Slyke is as follows: hemoglobin 3.6, fibrin 3.2, and lactalbumin 2.3. It is thus clearly evident that the error due to humin nitrogen in the analysis of these feedingstuffs is no greater than it is in the analysis of some of the pure proteins. From results recently obtained, we feel certain that the quantity of humin nitrogen formed in the hydrolysis of the nitrogenous constituents of feedingstuffs can be still further reduced.[2]

Briefly stated the details of the method used in this work are as follows: Weighed quantities of the feedingstuff are extracted with ether in Soxhlet extractors and then with cold absolute alcohol on Buchner funnels. The residues thus extracted are digested for 15 hours three or four times with 0.1 per cent solution of hydrochloric acid until all the starch has been converted into sugars. The residues insoluble in 0.1 per cent hydrochloric acid are boiled with 20 per cent hydrochloric acid until the proteins which they contain are completely hydrolyzed.

The filtrates from the residues insoluble in 0.1 per cent hydrochloric acid are neutralized with sodium hydroxide, then faintly acidified with acetic acid, allowed to stand over night, and then filtered. The filtrates from the precipitated proteins are concentrated *in vacuo* to small volumes and precipitated by the addition of five volumes of absolute alcohol. After standing over night, the precipitated proteins are removed by filtration and washed with 83 per cent alcohol.

The filtrates from the proteins precipitated by alcohol are concentrated to small volume and enough concentrated hydrochloric acid is added to make a 5 per cent solution. The solutions are then

[2] By improvement of the method reported here the humin content of corn has been still further reduced to 2.53 per cent since the manuscript for this paper was prepared.

boiled until hydrolysis is complete.[3] The proteins separated above by neutralization and by the addition of alcohol are boiled with 20 per cent hydrochloric acid until hydrolysis is complete.

As a result of the above procedure, there are obtained three different fractions of the proteins of feedingstuffs which are completely hydrolyzed; namely, (1) the proteins insoluble in 0.1 per cent hydrochloric acid; (2) the proteins separated by neutralizing the mineral acid and precipitating with alcohol; (3) the soluble proteins not separated by neutralizing the mineral acid or precipitating with alcohol. Each of the three hydrolyzed solutions is filtered and the insoluble humin substances are repeatedly digested with 0.1 per cent hydrochloric acid and then thoroughly washed with hot water. The nitrogen in these residues is considered to represent the insoluble humin substances.

The filtrates from the insoluble humin were concentrated *in vacuo* to small volume and the amide and soluble melanine nitrogen determined as usual. The remaining parts of the analysis are continued as usual. Only the results for the humin nitrogen are reported here; the complete analysis will be given in a later paper.

[3] In all cases it was found that a 5 per cent solution of hydrochloric acid would bring about the complete hydrolysis of the small amount of soluble proteins remaining in these solutions.

EXPERIMENTAL STUDIES ON GROWTH.

X. THE LATE GROWTH AND SENESCENCE OF THE NORMAL WHITE MOUSE, AND THE PROGRESSIVE ALTERATION OF THE NORMAL GROWTH CURVE DUE TO INBREEDING.

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Growth and Senescence of Normal White Mice.

In previous articles of this series[1] we have furnished data concerning the normal growth of the white mouse for the first 60 weeks of life, employing for the collection of these data a special technique designed to exclude accidental causes of fluctuation, such as epidemic infections, etc., and, as material for measurement, a large number of animals born in July, 1914, from a stock of animals purchased a few months previously from dealers.

Our observations upon these animals were continued until their death from natural causes, and weighings of every animal in the series were made fortnightly during the whole period of their existence (weekly during the first 30 weeks). We have now to report the complete series of measurements from the 4th week after birth until the termination of the observations by the death of the last surviving animal.[2] The results of these measurements are enumerated in Tables I and II and illustrated graphically in Figs. 1 and 2, displaying the progressive alterations of the average weight of the animals with increasing age.

[1] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347. Robertson, T. B., *ibid.*, 363.

[2] Data concerning growth from birth until the 4th week may be found in the articles cited above, also in Paper IX of this series (Robertson, T. B., and Delprat, M., *J. Biol. Chem.*, 1917, xxxi, 567).

An examination of these curves shows that the average weight of animals of both sexes increases, with a continuously decreasing velocity, to a definite maximum, attained at about 91 weeks of age in the male and at about 94 weeks of age in the female. After attaining this maximum the curve descends rather steeply, the loss of weight being much more rapid than the immediately preceding gain.

This loss of weight is the expression of senescence, and continues without interruption until the decease of the last surviving animal. It will be observed, however, that the curve of growth in both sexes displays marked oscillations towards the end, which are due to the irregularly occurring deaths of animals in which the process of senescence has been most rapid, and which have lost most weight. The survivors therefore represent an earlier or less complete stage of senescence than those which have died, and each group of late deaths is consequently accompanied by a rise in the weight curve of the survivors. Each rise, however, is succeeded by a fall whch is even more rapid than the preceding one, indicating that the process of senescence is in reality continuous, and moreover that it proceeds with a regularly increasing velocity depending upon the age rather than upon the weight of the animals. This general feature of the process of senescence is additionally illustrated by the curves accompanying Paper XI of the series.

The average duration of life (110 weeks in the male, 103 weeks in the female) exceeds by a definite interval the period during which the weight of the animals is increasing. The attainment of the average duration of life is therefore preceded by a decided measure of senescence, which must consequently have affected the viability of the majority of the animals and played a leading part in determining the mean duration of life. 78 per cent of the males and 68 per cent of the females survived the attainment of maximum weight, and therefore experienced the senescent loss of weight.

TABLE I.

Normal Males.

Age.	No. weighed.	Weight.	Variability.	Age.	No. weighed.	Weight.	Variability.
wks.		*gm.*	*per cent*	*wks.*		*gm.*	*per cent*
4	65	12.38	24.4	60	30	29.08	11.8
5	117	12.45	24.6	62	30	29.41	11.6
6	72	15.58	22.0	64	30	29.53	11.4
7	43	18.08	16.9	66	30	29.70	11.4
8	42	19.36	15.9	68	30	29.80	11.1
9	42	20.63	16.5	70	30	29.70	10.9
10	42	21.19	16.7	72	30	29.70	12.2
11	42	21.81	13.3	74	29	29.76	10.9
12	42	22.65	13.7	76	29	29.98	10.0
13	37	23.31	14.2	78	29	30.16	11.5
14	36	23.96	14.1	80	29	29.95	11.1
15	36	24.28	11.9	82	26	30.31	11.3
16	36	24.75	12.7	84	26	29.88	12.3
17	36	25.21	12.4	86	25	30.10	13.0
18	36	25.61	12.1	88	24	30.19	13.0
19	35	25.81	12.1	90	24	30.13	11.3
20	35	26.10	10.8	92	24	30.46	12.0
21	35	26.28	11.2	94	24	30.13	11.1
22	35	26.06	9.0	96	23	29.67	10.1
23	34	26.34	9.8	98	22	29.36	10.0
24	34	26.82	10.1	100	21	29.54	9.6
25	33	27.05	11.0	102	20	29.48	10.7
26	33	26.94	10.8	104	19	29.61	10.4
27	32	26.55	11.3	106	16	29.03	10.7
28	31	27.19	10.9	108	16	28.56	9.6
29	31	27.08	10.9	110	16	28.25	11.0
30	31	27.23	9.5	112	15	28.30	11.3
32	32	27.61	9.6	114	16	28.16	11.3
34	31	27.71	10.5	116	13	28.19	12.6
36	30	27.95	10.1	118	14	28.29	12.8
38	30	28.20	10.5	120	12	27.25	10.9
40	30	28.27	10.4	122	12	27.08	10.6
42	30	28.28	11.2	124	11	27.32	10.2
44	30	28.62	12.0	126	11	26.73	12.3
46	30	28.55	11.7	128	10	27.55	10.6
48	30	28.22	10.8	130	9	28.11	10.8
50	30	28.88	11.1	132	5	28.00	11.2
52	30	28.68	10.7	134	5	28.00	—
54	30	28.67	12.0	136	5	27.50	—
56	30	29.32	12.0	138	2	25.50	—
58	30	29.10	10.9	140	1	26.00	—

TABLE II.

Normal Females.

Age.	No. weighed.	Weight.	Variability.	Age.	No. weighed.	Weight.	Variability.
wks.		*gm.*	*per cent*	*wks.*		*gm.*	*per cent*
4	89	10.39	23.4	66	28	26.45	19.2
5	77	11.81	19.4	68	28	26.55	18.3
6	68	14.12	18.9	70	27	26.13	18.7
7	37	16.77	15.0	72	26	26.56	17.7
8	36	17.99	13.9	74	25	26.76	16.9
9	36	18.78	13.4	76	25	27.24	17.7
10	36	19.38	14.3	78	23	27.39	17.0
11	36	20.04	12.6	80	22	27.64	17.4
12	36	20.31	12.6	82	22	27.39	17.0
13	36	21.04	13.0	84	22	27.75	16.4
14	36	21.21	13.2	86	22	27.89	17.6
15	36	21.78	13.7	88	22	27.77	18.7
16	36	22.14	12.6	90	22	27.82	16.8
17	36	22.29	11.9	92	21	27.98	18.4
18	36	22.22	11.9	94	20	27.53	18.0
19	36	22.60	12.0	96	20	28.03	17.8
20	36	22.60	11.2	98	19	27.89	20.9
21	36	23.06	12.3	100	19	27.42	22.2
22	36	23.32	12.5	102	17	27.62	20.7
23	35	23.51	12.0	104	16	27.06	19.1
24	36	23.68	11.3	106	15	26.57	18.6
25	35	23.79	11.4	108	11	26.82	18.2
26	36	24.04	11.6	110	10	26.30	17.7
27	35	24.00	11.8	112	10	26.70	16.0
28	36	23.58	10.3	114	7	24.43	10.5
29	35	23.84	12.2	116	7	24.57	9.7
30	36	23.92	12.4	118	7	24.29	10.0
32	36	24.18	11.5	120	7	24.14	10.0
34	36	24.18	12.2	122	7	24.93	11.0
36	36	24.65	11.2	124	7	24.93	9.5
38	36	24.80	12.3	126	6	24.83	9.8
40	35	25.03	11.9	128	5	25.80	7.8
42	35	25.07	13.1	130	5	25.80	8.1
44	34	25.52	15.3	132	5	25.10	6.6
46	33	25.68	14.6	134	5	25.50	7.8
48	32	25.45	14.6	136	4	24.00	—
50	32	25.50	14.0	138	4	24.50	—
52	29	25.76	15.3	140	4	24.63	—
54	29	25.78	15.8	142	4	24.88	—
56	29	26.00	16.5	144	3	24.00	—
58	29	26.26	16.7	146	3	24.50	—
60	29	26.12	17.8	148	2	24.00	—
62	29	26.40	17.7	150	2	24.00	—
64	29	26.64	17.8				

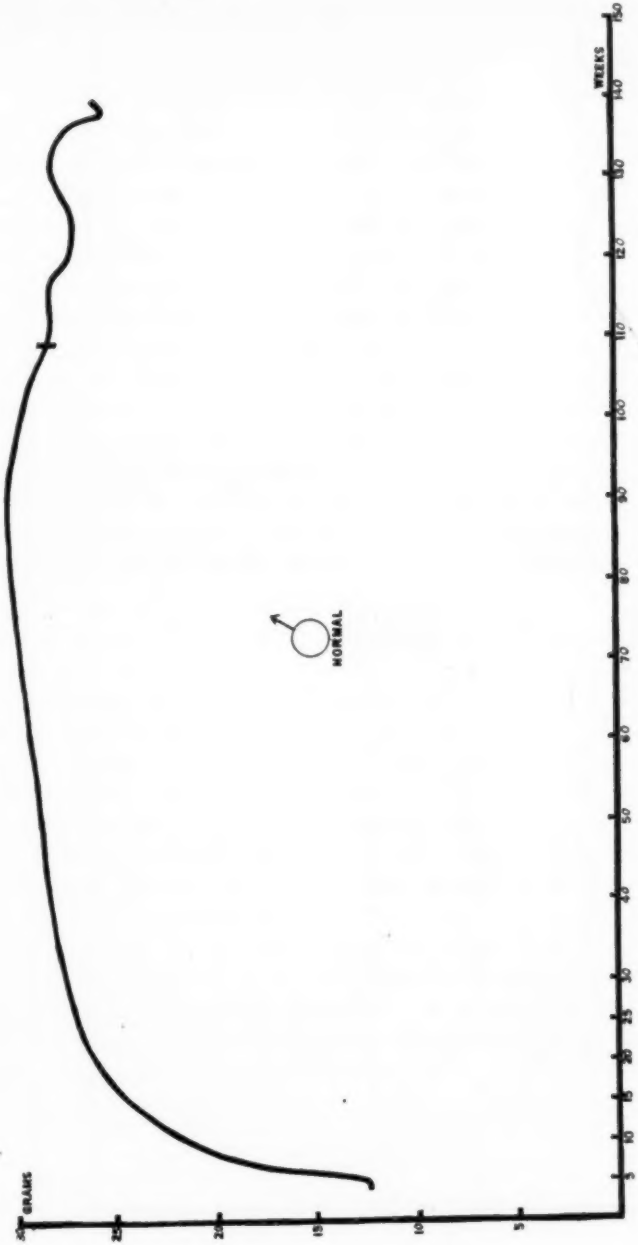

FIG. 1. Growth curve of normal male white mice from 4 weeks until death of the last surviving animal. The vertical cross mark indicates average duration of life.

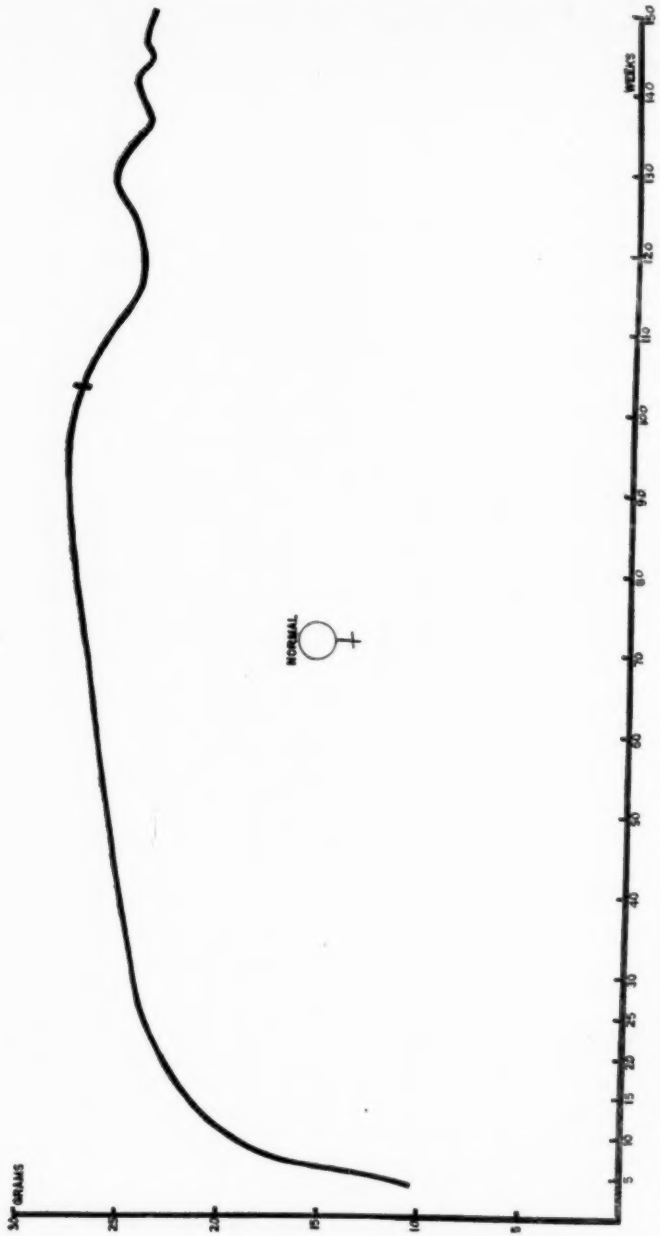

FIG. 2. Growth curve of normal female white mice from 4 weeks until death of the last surviving animal. The vertical cross mark indicates average duration of life.

Progressive Alteration of Normal Growth Curve Due to Inbreeding of Mixed Stock.

Our original stock of animals was obtained from animal dealers, and therefore consisted in all probability of a variety of different strains. We have consistently adopted a plan of rotation whereby it is assured that no female is ever impregnated twice in succession by the same male. In this way thorough mixture of the strains has no doubt been brought about in the course of time, and close inbreeding has been avoided. We have found, however, that successive batches of animals thus obtained display definite and progressive changes in the curve of growth.

Since the changes in question are quantitative, and do not exceed the individual differences which may frequently be observed between the weights of any single pair of animals selected at random, the only method of attaining any degree of certainty as to the reality of the differences is to determine the ratio of the observed average differences between successive batches of normals to the probable error of the experimental estimate of the differences themselves; that is, to $\sqrt{E_1^2 + E_2^2}$ where E_1 and E_2 are the probable errors of the separate estimates of average weight.[3]

The chance of any given, observed deviation being accidental may be estimated from Table III, computed from a table of areas of the normal frequency curve.[4] Thus if the ratio of an observed deviation from the standard chosen to the probable error of its estimation is 3.0, then there is one chance that it is accidental to 22 that it is not accidental but systematic in origin; *i.e.*, that it originates in a definite and not fortuitous difference between the two groups of animals investigated.

In Tables IV to VII and Figs. 3 and 4 are given the comparative growth of three successive batches of normal males, born, respectively, in July 1914, November 1914, and July 1916, and of three successive batches of normal females born, respectively, in July 1914, July 1916, and October 1917. The curves reveal a

[3] Davenport, C. B., Statistical methods with special reference to biological variation, New York, 2nd edition, 1904, 15.

[4] Davenport, C. B.,[3] p. 119.

regularly diminishing velocity and absolute magnitude of growth in each succeeding batch of animals.

The absolute difference between the average weights of July and November 1914 males, averaging from 1.0 to 1.5 gm., is only slightly less than that between the males of November 1914 and July 1916 (1.5 to 2.0 gm.), although a period of 20 months elapsed between the birth of the two latter groups and of less than 5 months between the birth of the two former groups. Nearly as much change in the stock occurred, therefore, in the first 5 months of inbreeding as in the succeeding 20 months.

Owing to the much smaller variability of the animals comprising the later batches, however, the observed deviations only average from two to three times the experimental error for the 1914 batches, while they average between four and six times the experimental error in the case of the November 1914 and July 1916 batches of animals. In both sexes diminution of the variability of the animals, as in other instances previously noted,[5] is associated with diminished rapidity of growth.

The effects are much smaller in the females, the differences between the 1917 and 1916 batches lying almost entirely within the experimental error of the estimations.

It would appear, therefore, that the animals are approaching, if they have not already reached, a stable type, but that this requires a considerable period of time notwithstanding the deliberate effort made to secure thorough mixing of the strains. From these facts it will be evident that no growth standard, no matter upon how many observations it may be established, is applicable to animals derived from a different strain or stock. Each stock must provide its own standard norm and this norm must be reestablished at frequent intervals.

[5] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 372; *Am. J. Physiol.*, 1916, xli, 535, 547. Thompson, D. W., Growth and form, Cambridge, 1917.

TABLE III.

Ratio of deviation from normal to probable error of estimate.	Chance that observed deviation is accidental.	Ratio of deviation from normal to probable error of estimate.	Chance that observed deviation is accidental.
0.1	18 to 1	2.8	1 to 16
0.2	7.5 " 1	2.9	1 " 19
0.3	5.2 " 1	3.0	1 " 22
0.4	3.7 " 1	3.1	1 " 26
0.5	2.8 " 1	3.2	1 " 31
0.6	2.2 " 1	3.3	1 " 37
0.7	1.7 " 1	3.4	1 " 45
0.8	1.4 " 1	3.5	1 " 54
0.9	1.2 " 1	3.6	1 " 65
1.0	1 " 1	3.7	1 " 79
1.1	1 " 1.2	3.8	1 " 95
1.2	1 " 1.4	3.9	1 " 116
1.3	1 " 1.6	4.0	1 " 143
1.4	1 " 1.9	4.1	1 " 172
1.5	1 " 2.2	4.2	1 " 214
1.6	1 " 2.5	4.3	1 " 266
1.7	1 " 2.9	4.4	1 " 335
1.8	1 " 3.4	4.5	1 " 415
1.9	1 " 4.0	4.6	1 " 514
2.0	1 " 4.6	4.7	1 " 657
2.1	1 " 5.4	4.8	1 " 832
2.2	1 " 6.3	4.9	1 " 1,041
2.3	1 " 7.3	5.0	1 " 1,310
2.4	1 " 8.5	6.0	1 " 19,200
2.5	1 " 9.9	7.0	1 " 420,000
2.6	1 " 11.6	8.0	1 " 17,000,000
2.7	1 " 14		

TABLE IV.

Normal Males, November 1914.

Age.	No. weighed.	Weight.	Deviation from normal of July 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
5	15	14.63	+2.18	3.3	24.6
6	24	15.54	−0.04	0.1	19.7
7	24	16.77	−1.31	2.6	17.1
8	24	17.92	−1.44	3.1	13.9
9	24	17.92	−2.71	5.3	15.1
10	24	19.10	−2.09	4.2	12.6
11	24	19.06	−2.75	6.0	13.4
12	24	20.46	−2.19	4.4	13.7
13	24	21.38	−1.93	3.7	12.3
14	24	22.58	−1.38	2.7	10.8
15	24	23.23	−1.05	2.4	9.2
16	24	23.73	−1.02	2.3	8.8
17	24	24.23	−0.98	2.2	7.8
18	23	24.48	−1.13	2.6	7.3
19	24	24.71	−1.10	2.4	8.3
20	23	25.15	−0.95	2.3	7.2
21	23	25.04	−1.24	3.0	7.2
22	23	25.61	−0.45	1.2	6.8
23	23	25.35	−0.99	2.6	6.8
24	23	25.65	−1.17	2.9	7.0
25	23	26.02	−1.03	2.4	6.4
26	22	25.89	−1.05	2.4	7.2
27	23	25.70	−0.85	1.9	7.4
28	22	26.14	−1.05	2.3	7.1
29	23	26.09	−0.99	2.2	7.4
30	18	26.06	−1.17	2.7	7.3
32	23	26.72	−0.89	2.1	7.4
34	23	26.48	−1.23	2.7	7.6
36	23	26.54	−1.41	3.1	7.5
38	23	26.91	−1.29	2.8	7.5
40	23	27.17	−1.10	2.3	7.8
42	23	27.76	−0.52	1.1	7.5
44	23	27.85	−0.77	1.5	7.6
46	23	27.85	−0.70	1.4	7.5
48	22	28.11	−0.11	2.3	7.3
50	22	28.00	−0.88	1.8	7.9
52	22	28.02	−0.66	1.3	7.9
54	22	27.84	−0.83	1.6	7.6
56	20	27.85	−1.47	2.8	8.6
58	16	28.00	−1.10	2.1	7.6
60	16	28.19	−0.89	1.6	7.7

TABLE V.

Normal Males, July 1916.

Age.	No. weighed.	Weight.	Deviation from normal of Nov. 1914.	Times probable error of difference.	Variability
wks.		*gm.*			*per cent*
5	20	13.25	−1.38	1.9	19.2
6	24	15.04	−0.50	0.9	15.8
7	24	16.83	+0.06	0.1	13.7
8	24	18.08	+0.16	0.4	12.1
9	24	19.00	+1.08	2.3	11.9
10	24	19.63	+0.53	1.2	10.6
11	24	19.96	+0.90	1.9	11.9
12	24	20.44	−0.02	0.0	11.5
13	24	20.92	−0.46	0.9	12.1
14	24	21.31	−1.27	2.7	11.3
15	24	21.83	−1.40	3.0	11.6
16	24	22.15	−1.58	3.5	11.5
17	24	22.42	−1.81	4.1	11.7
18	24	22.58	−1.90	4.5	10.9
19	24	22.71	−2.00	4.6	10.7
20	24	23.02	−2.13	5.2	10.5
21	24	23.10	−1.94	4.8	9.1
22	24	23.42	−2.19	5.3	9.8
23	24	23.49	−1.86	4.6	9.8
24	24	23.63	−2.02	5.3	8.8
25	24	23.85	−2.17	6.0	8.3
26	24	24.10	−1.79	4.4	9.5
27	24	24.31	−1.39	3.6	8.5
28	22	24.31	−1.83	4.5	8.8
29	24	24.65	−1.44	3.6	8.5
30	17	24.91	−1.15	2.5	8.6
32	24	24.88	−1.84	4.5	8.9
34	24	25.21	−1.27	3.0	8.7
36	24	25.69	−0.85	2.1	8.3
38	24	25.52	−1.39	3.3	8.8
40	24	25.83	−1.34	3.1	8.8
42	24	26.00	−1.76	4.2	8.3
44	24	26.06	−1.79	4.3	8.3
46	24	26.56	−1.29	3.1	8.2
48	24	26.35	−1.76	4.1	8.5
50	23	26.85	−1.15	2.6	8.2
52	23	27.20	−0.82	1.8	8.3
54	23	27.61	−0.23	0.5	8.7
56	23	27.54	−0.31	0.6	8.0
58	23	27.57	−0.43	0.9	8.7
60	23	27.39	−0.80	1.7	7.5

TABLE VI.

Normal Females, 1916.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	15	12.43	+2.04	6.2	12.9
5	24	13.67	+1.86	6.0	13.2
6	24	15.08	+0.96	2.6	13.9
7	24	15.98	−0.79	2.1	10.8
8	24	16.94	−1.05	3.0	9.0
9	24	17.50	−1.28	3.6	9.7
10	24	17.73	−1.65	4.6	8.0
11	24	18.19	−1.85	4.9	9.8
12	24	18.42	−1.89	5.1	9.0
13	24	18.56	−2.48	6.4	8.8
14	24	19.23	−1.98	4.9	9.5
15	24	19.42	−2.36	5.6	9.8
16	24	19.90	−2.24	5.6	9.5
17	24	20.19	−2.10	5.1	10.1
18	24	20.06	−2.16	5.4	9.3
19	24	20.31	−2.29	5.6	9.6
20	24	20.88	−1.72	4.4	9.7
21	23	20.85	−2.21	5.0	10.2
22	23	20.89	−2.43	5.5	9.8
23	23	21.22	−2.29	5.1	10.7
24	23	21.39	−2.29	5.3	10.6
25	23	21.59	−2.20	4.6	12.0
26	23	21.76	−2.28	4.5	13.1
27	23	22.00	−2.00	3.9	13.1
28	21	22.71	−0.87	1.8	11.6
29	22	22.77	−1.07	2.0	13.1
30	21	22.83	−1.09	2.2	11.4
32	23	23.11	−1.07	2.1	12.3
34	23	23.17	−1.01	2.0	11.4
36	23	23.65	−1.00	2.0	11.4
38	23	23.80	−1.00	1.8	12.9
40	23	23.93	−1.10	2.0	13.1
42	23	24.48	−0.59	1.0	14.2
44	23	24.00	−1.52	2.4	13.3
46	23	24.24	−1.44	2.5	11.2
48	23	24.07	−1.38	2.4	11.2
50	23	24.04	−1.46	2.6	11.1

TABLE VII.

Normal Females, 1917.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	24	10.27	−2.16	5.3	21.4
5	24	12.15	−1.52	3.7	20.0
6	24	13.73	−1.35	3.3	15.2
7	24	14.96	−1.02	2.7	14.2
8	24	15.29	−1.65	4.7	13.3
9	23	16.28	−1.22	3.4	12.1
10	22	16.64	−1.07	3.1	11.5
11	22	17.39	−0.80	2.2	11.0
12	22	17.75	−0.67	1.8	11.3
13	22	18.36	−0.20	0.6	10.3
14	22	18.61	−0.62	1.6	11.0
15	22	19.05	−0.37	0.9	11.0
16	22	18.80	−1.10	2.4	13.9
17	22	19.55	−0.64	1.5	11.0
18	22	19.66	−0.40	1.0	11.0
19	22	20.05	−0.26	0.6	11.0
20	22	20.59	−0.29	0.7	10.2
21	22	21.05	+0.20	0.5	9.5
22	22	21.48	+0.59	1.4	9.8
23	22	21.70	+0.48	1.1	9.0
24	22	21.68	+0.29	0.7	9.1
25	22	22.25	+0.66	1.3	10.1
26	22	22.77	+1.01	1.9	10.2
27	21	22.81	+0.81	1.6	10.0
28	22	22.70	−0.01	0.0	9.6
29	21	23.24	+0.47	0.8	11.3
30	21	23.71	+0.88	1.7	11.0
32	22	24.16	+1.05	1.8	11.4
34	22	24.23	+1.06	2.0	10.8
36	22	24.50	+0.85	1.5	12.3
38	5	24.50	+0.70	—	—
40*	18	23.19	−0.74	1.2	11.4
42	17	23.59	−0.89	1.5	9.2
44	17	24.47	+0.47	0.9	7.1
46	19	24.89	+0.65	1.2	10.2
48	19	25.13	+1.06	1.9	10.4
50	19	25.53	+1.49	2.9	9.1

* Moved from California to Toronto.

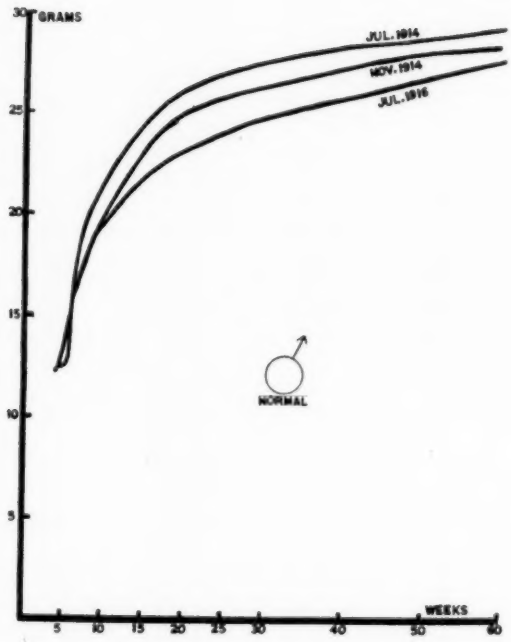

FIG. 3. Progessive changes in the growth curve of normal male white mice from July 1914 to July 1916.

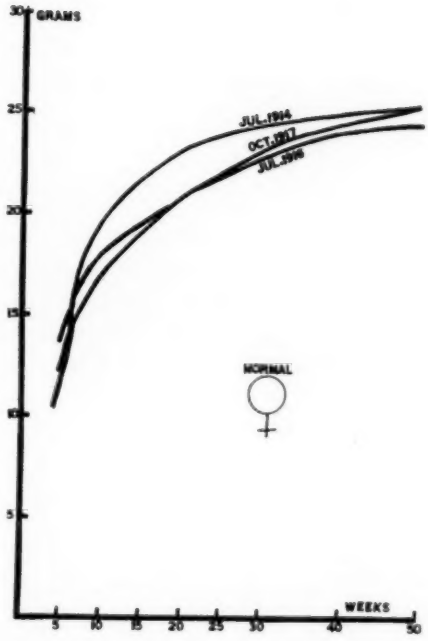

FIG. 4. Progessive changes in the growth curve of normal female white mice from July 1914 to October 1917.

EXPERIMENTAL STUDIES ON GROWTH.

XI. THE GROWTH AND SENESCENCE OF WHITE MICE FED UPON PITUITARY (ANTERIOR LOBE) TISSUE, TETHELIN, EGG LECITHIN, OR CHOLESTEROL.

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General Characteristics of Effects Observed.

In preceding articles of this series[1] data were presented concerning the growth of animals receiving (superadded to a varied and abundant normal diet) specified amounts of pituitary (anterior lobe) tissue, tethelin,[2] egg lecithin, or cholesterol. The results reported at that time extended only to the 60th week of life and the 56th week of the administration of the substances added to the diet. The conclusions reached were: (1) the pituitary (anterior lobe) tissue in dosage of one-twelfth of an anterior lobe of the ox per mouse per day causes a retardation of growth during the first part of the third or adolescent growth cycle, followed by a secondary or compensatory acceleration; (2) tethelin in dosage of 4 mg. per mouse per day leads to similar results of a much more pronounced character and extent; (3) egg lecithin in dosage of 40 mg. per mouse per day causes a uniform retardation of growth probably originating in a certain degree of toxicity of the very large dose of lecithin or of the choline split off from it during digestion; (4) cholesterol in dosage of 40 mg. per mouse per day causes a pronounced initial retardation and sec-

[1] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385, 397; xxv, 635, 647.

[2] Concerning the preparation, properties, and actions of tethelin, a phospholipin derived from the anterior lobe of the pituitary body, see: Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 409; *Endocrinology*, 1917, i, 24. Schmidt, C. L. A., *J. Lab. and Clin. Med.*, 1916–17, ii, 711. Schmidt, C. L. A., and May, E. S., *ibid.*, 1916–17, ii, 708.

ondary acceleration similar in type to that produced by much smaller doses of tethelin.

In the previous reports, however, only the absolute magnitudes of the observed deviations from normality were considered, and no estimate of their real significance in terms of the probable error of the measurements was furnished. In the present experiments the results previously published have been amplified by continuing the observations from the 60th week to natural death, and the observed deviations from the normal have been computed in the only manner which enables us to determine the precise de-

TABLE I.

Summary of Tables II to IX.

Class of animals.	Sex.	Average ratio of deviation from normal to probable error of estimate.			
		Weeks.			
		4-30	32-60	62-90	92-120
Pituitary.....	♂	2.1	0.8	1.1	1.8
"	♀	4.0	1.1	0.6	1.2
Tethelin.....	♂	4.2	3.9	3.2	1.0
"	♀	3.4	2.0	2.7	2.2
Lecithin.....	♂	5.0	2.5	4.7	4.5
"	♀	5.2	3.3	3.3	2.7
Cholesterol...	♂	5.7	3.9	4.5	4.0
"	♀	6.5	2.5	3.2	3.3

gree of significance they possess; namely, in terms of the probable error of the estimation of the deviations themselves.[3]

These extended results confirm in general the conclusions set forth in the earlier reports. They are summarized in Table I, and are shown in detail in Tables II to IX and Figs. 1 to 8.

The greater the value of the ratio of the observed deviations from the normal to the probable error of their estimation, the greater is the significance which attaches to them. From Table III of the preceding article, it will be seen that a deviation which is twice the probable error of the estimation has one chance in

[3] Paper X of this series.

5.6 of being accidental, while a deviation which is six times the probable error of the estimation has only one chance in over 19,000 of being accidental in origin. A ratio of less than one indicates that the observed deviation from the normal is more probably accidental than not.

Table I shows that in all classes of animals, whether receiving pituitary tissue, tethelin, lecithin, or cholesterol, the deviations from the normal expressed in probable error units are greatest in the first 30 weeks and thereafter diminish progressively. Evidently all the different classes of animals of the same sex tended to approach a common curve of final equilibrium, the attainment of which was only prevented by the onset of senescence and the loss of weight and mortality which ensue. The substances administered to the animals, therefore, influenced the growth process in the way that catalyzers influence chemical reactions; that is, by affecting the velocity with which equilibrium is attained without affecting the equilibrium itself.

In all instances, except one, the effect of the substance added to the dietary was, during the first 30 weeks, much greater in the females than in the males. The sole exception is that afforded by the tethelin-fed animals in which the effect upon the males was greater than upon the females. This exception affords striking testimony to the reality of the effects observed, for tethelin was only administered to the females for three periods of 1 month each (see Fig. 4), while it was administered to the males daily, without interruption.

Effects of Pituitary (Anterior Lobe) Tissue.

The computation of the deviations in terms of probable error units shows that the effect of the dosage of pituitary tissue administered upon the growth of the male animals was of uncertain significance, since the observed deviations were only from one to two times the probable error. That the deviations from the normal nevertheless were real and due to the administration of the pituitary tissue is evidenced by the much greater effect of the same character upon the female, consisting of retardation during the earlier stages of growth. In both males and females the deviations from the normal after the 30th week are of indeterminate

significance; that is, the growth curves of the normal and of the pituitary-fed animals are, so far as our estimates reveal, identical after 7 months of age. Hence the preliminary retardation of growth has clearly, prior to the 30th week, been succeeded by acceleration.

Effects of Tethelin.

The dosage of pituitary tissue which was administered to the pituitary-fed animals corresponded to a dosage of only about 0.8 mg. of tethelin *per diem*. The effect of 4 mg. of tethelin per day is much more marked and consists of initial retardation followed by marked secondary or compensatory acceleration. The effects are definite in magnitude and remain so until the last period of 92 to 120 weeks (see Table I) when the diminishing number of survivors in both the normal and experimental groups so enhances the probable error of the estimations that marked deviations are requisite for significance.

It remains to inquire whether the acceleration of growth succeeding the 15th week in the tethelin-fed groups is due to the tethelin directly or only indirectly; whether it is due to direct stimulation of the growth process by the tethelin, or to some compensatory process started in the animal itself by the administration, and as a protection against the retarding action of the tethelin.

In our previous reports we subscribed to the former alternative, but we now incline to the latter since we find that by discontinuing the administration of tethelin before the attainment of sexual maturity of the animals the acceleration not only still occurs, but is enhanced. In fact, we have succeeded by this method in producing some very large animals; namely, a number of females weighing over 35 and even over 40 gm. each. The details of these results will be reported later but an idea of their character may be obtained from the curves in Paper XIV of this series.

Effects of Egg Lecithin.

In our earlier reports we considered egg lecithin to be without direct influence upon the growth processes, for the reason, primarily, that the curves of growth of the lecithin-fed animals,

although below normal, remained parallel to the normal curves throughout the first 60 weeks without exhibiting the decisive deformations of contour characteristic of the growth curves of tethelin-fed or cholesterol-fed animals. Our present method of evaluating the results shows that our previous conclusion was incorrect and at the same time reveals the value of the statistical method when applied to investigations of this character.

The deviations from normality in the first 30 weeks are of such magnitude that there is but one chance in 1,300 that they were accidental, nor could any initial error in sampling the material from which the experimental animals were chosen have produced these deviations, for during the first few weeks of lecithin dietary the deviations from the normal increased from three to six times the probable error in the males, and from 0.5 to six times the probable error in the females.

The deviation from normality, although diminishing somewhat (in probable error units) with advancing age, remained significant throughout life.

We incline to the view that the effects of egg lecithin in dosage of 40 mg. daily are of a twofold origin. That there is probably some toxicity is indicated by the indifferent appearance of the animals (described in our previous reports), by the subnormal duration of life (cf. Paper XII), and by their remarkable susceptibility to infectious and degenerative lesions (cf. Paper XIII). This is confirmed by the marked tendency (nearly always present, but especially prominent in the lecithin-fed animals) to abmodality in the frequency-curve of mortality; *i.e.*, the marked divergence between the mean and the median durations of life (cf. Paper XII). The scattering of deaths is not uniform on either side of the greatest frequency, but, on the contrary, the 50 per cent of animals which survive the median duration of life survive to a greater age than we should anticipate from the distribution of the earlier deaths. Hence a small proportion of lecithin-fed animals, notwithstanding the subnormal duration of life of the average, survived to what would constitute a very advanced age even in normal animals.

The egg lecithin employed was a crude preparation, obtained by simply precipitating the ether extract of egg yolks with acetone, draining, and drying without reprecipitation. It must

therefore have consisted of a mixture of lipins. We are inclined to refer the toxicity of the material to the lecithin itself, administered in excessively large dosage, while in view of the crude character of the preparation used and the ease with which lecithin itself is known to be saponified and the consequent unlikelihood of its absorption without alteration, we are inclined to attribute its effects upon the time relations of growth to a substance or substances associated with our preparations, rather than to the lecithin itself.

Effects of Cholesterol.

The effects of cholesterol in dosage of 40 mg. daily are the most marked, and consist, as previously stated, in decided initial retardation followed by acceleration of growth. The deviations from normal are significant throughout life and could not have arisen accidentally.

Possible Errors of Sampling.

The foundation upon which statistical comparisons are based is the assumption, the correctness of which must be verified in each particular instance, that the material subjected to measurement is uniformly variable about a fixed mean, so that successive samples differ from one another only fortuitously and not in consequence of any systematic cause of variation or shift in value of the mean. Now as we have seen in the preceding article the normal animals from which the experimental animals were chosen were varying[3] and the mean weight at every age was changing with the lapse of time. It was impossible to start all these feeding experiments simultaneously, involving as they did the use of over 500 animals, and therefore a systematic source of error underlies the results and it becomes necessary to estimate the degree to which it may have affected their verity.

The experiments were begun in the following order: normals (born July 1914), pituitary (born July 1914), lecithin (born August 1914), tethelin, males (born August 1914), cholesterol (born October 1914), tethelin, females (born October 1914). In November to December 1914 a fresh batch of normal males was chosen, which, as explained in the preceding article, were found to

differ from the July normals by deviations averaging only about twice the probable error of the estimate. Moreover, their growth curve showed no trace of deformation of contour such as the concavity between 10 and 20 weeks which characterizes the growth curves of tethelin and cholesterol-fed animals. Quantitatively and qualitatively, therefore, the observed effects could only have been affected by errors of sampling to an insignificant degree.

The reality of the effects observed is, however, furthermore established, in a striking way, by the fact that in all the experiments the effect of the administration (except for the reasons noted above, in the tethelin group) was much greater in the females than in the males. The progressive alteration of the normal with time was very much *less*[3] in the female than in the male. The result is therefore the opposite of what it would have been had the observed divergences from normality been due to errors in sampling the material used for measurement.

Effects upon Variability.

The effects of the various substances administered upon the variability of the animals were diverse. In all classes of animals and in both sexes the variability during the early months of growth was depressed in proportion to the diminution of the velocity of growth. In later months, with the exception of the cholesterol-fed groups, the variability of the males remained subnormal, while that of the females regained the normal level or even exceeded it. Exceptions, as stated, were afforded by both sexes of the cholesterol-fed group, which, despite their subnormal weight, became abnormally variable in weight after the first 6 or 7 months. This high variability was probably attributable in part to the disturbances of function arising from extensive deposits of cholesterol in various organs,[4] and the degenerative lesions which resulted

[4] Anitschkow, N., *Beitr. path. Anat. u. allg. Path.*, 1913, lvi, 379; 1914, lvii, 201. Chalatow, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 85. Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 68; 1915–16, xiii, 60.

The presence of extensive deposits of doubly refracting lipoids in the liver and adrenals of these animals was confirmed by Dr. C. H. Bailey, to whom our thanks are due for examining many specimens of the material.

therefrom in later months (Paper XIII). It has been pointed out in connection with the growth of children[5] that subnormal velocity of growth, when accompanied by deleterious conditions, leads to enhanced variability, while under favorable conditions it leads to subnormal variability.

In all the different classes of animals, normal as well as experimental, a tendency was displayed for a small group of similar animals to survive to the last weeks. In other words, a certain small number of animals which resembled each other in surviving longest also resembled each other in weight, as though a certain rather definite weight standard, differing in each class of animals, were the most favorable for survival.

Effects upon Senescence.

In all the growth curves (Figs. 1 to 8) senescent loss of weight precedes by a definite interval the average duration of life, and the terminal oscillations of the curve reveal successively steeper descending limbs, showing that senescent loss of tissue is proceeding with augmenting velocity as age increases. The position of the maximum of the growth curve is, however, considerably affected by the administrations, and almost disappears in the curve of the tethelin males (which received tethelin continuously) and in the curves of the lecithin-fed animals. The hump on the normal curve is replaced in these cases by a long, flattened plateau, gradually rising (tethelin males) or falling (lecithin males and females) till the relatively rapid fall due to senescence supervenes. It is probably significant that the greatest flattening of the curve (*i.e.*, slowing of the rate of change of growth velocity) occurred in precisely those groups of animals which displayed the greatest duration of life (tethelin males), or else in which certain exceptional individuals displayed a remarkably enhanced duration of life (lecithin males).

[5] Robertson, T. B., *Am. J. Physiol.*, 1916, xli, 547.

TABLE II.

Pituitary-Fed Males.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	20	12.78	+0.40	0.8	22.7
5	36	13.13	+0.68	1.7	24.1
6	36	15.86	+0.28	0.6	22.1
7	36	17.38	−0.70	1.5	17.7
8	36	18.60	−0.76	1.6	17.3
9	36	19.54	−1.09	2.3	14.8
10	36	20.03	−1.16	2.3	15.4
11	36	20.58	−1.23	2.7	14.9
12	36	21.79	−0.86	2.0	11.4
13	36	22.36	−0.95	2.1	10.7
14	36	23.00	−0.96	2.1	10.0
15	36	23.06	−1.22	3.0	9.8
16	36	23.67	−1.08	2.5	9.3
17	36	24.11	−1.10	2.5	9.9
18	36	24.17	−1.44	3.3	9.7
19	36	24.57	−1.24	2.8	10.2
20	36	24.63	−1.47	3.5	9.8
21	36	24.99	−1.29	3.0	9.6
22	36	25.35	−0.71	1.7	11.5
23	36	25.85	−0.49	1.3	8.7
24	36	26.19	−0.63	1.4	10.5
25	36	25.92	−1.13	2.5	9.8
26	36	26.11	−0.83	1.8	9.2
27	36	26.11	−0.44	1.0	8.8
28	34	26.29	−0.90	2.0	8.7
29	35	26.23	−0.85	1.9	8.8
30	33	26.48	−0.75	1.9	8.4
32	36	26.68	−0.93	2.2	9.0
34	34	27.35	−0.36	0.8	9.7
36	32	27.33	−0.62	1.4	9.3
38	32	27.55	−0.65	1.4	9.3
40	32	28.03	−0.24	0.5	9.2
42	31	28.05	−0.23	0.4	10.6
44	30	28.32	−0.30	0.6	10.0
46	31	28.24	−0.31	0.6	10.1
48	30	28.05	−0.17	0.3	11.1
50	30	28.47	−0.41	0.8	11.2
52	30	28.58	−0.10	0.2	11.2
54	30	28.92	+0.25	0.4	11.4
56	30	28.90	−0.42	0.7	11.1
58	30	29.35	+0.25	0.5	11.0
60	30	29.42	+0.34	0.6	11.0

TABLE II—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
62	30	29.12	−0.29	0.5	10.6
64	30	29.30	−0.23	0.4	11.3
66	30	29.80	+0.10	0.2	11.4
68	30	29.63	−0.17	0.3	11.4
70	28	29.29	−0.41	0.7	11.9
72	28	29.43	−0.27	0.5	11.0
74	28	29.64	−0.12	0.2	11.5
76	28	29.21	−0.77	1.4	11.2
78	27	29.41	−0.75	1.1	10.2
80	27	29.02	−0.93	1.7	9.8
82	27	29.04	−1.27	2.2	10.1
84	27	29.33	−0.55	0.9	10.2
86	26	28.44	−1.66	2.7	8.2
88	25	28.82	−1.37	2.1	9.0
90	25	28.90	−1.23	2.1	8.6
92	24	28.04	−2.42	4.3	6.5
94	24	28.44	−1.69	3.1	7.9
96	24	28.29	−1.38	2.7	7.8
98	23	28.17	−1.19	2.2	8.4
100	23	28.24	−1.30	2.5	8.1
102	21	28.31	−1.17	1.9	9.2
104	21	27.90	−1.71	3.0	7.5
106	21	28.00	−1.03	1.7	8.1
108	17	27.94	−0.62	1.1	6.7
110	16	27.91	−0.34	0.5	7.6
112	13	28.46	+0.16	0.3	6.3
114	12	27.29	−0.87	1.2	9.1
116	11	27.32	−0.87	1.1	6.8
118	11	27.45	−0.84	1.1	6.7
120	11	27.59	+0.34	0.5	6.3
122	11	27.23	+0.15	0.2	6.2
124	11	27.00	−0.32	0.5	6.6
126	10	26.95	+0.22	0.3	7.9
128	7	26.93	−0.62	0.8	7.9
130	6	25.75	−2.36	2.9	6.5
132	6	26.00	−2.00	1.8	8.1
134	6	25.67	−2.33	—	8.1
136	5	24.90	−2.10	—	7.2
138	4	25.38	−0.12	—	—
140	4	25.13	−0.87	—	—
142	2	26.25	—	—	—
144	2	26.00		—	—
146	2	25.50		—	—
148	1	26.50	—	—	—

TABLE III.

Pituitary-Fed Females.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	31	9.53	−0.86	3.1	20.5
5	35	11.76	−0.05	0.2	19.6
6	35	13.89	−0.23	0.7	16.1
7	36	14.71	−2.06	5.4	15.8
8	36	16.24	−1.75	4.5	15.1
9	36	17.22	−1.56	4.1	13.1
10	36	17.93	−1.45	3.7	12.1
11	36	18.24	−1.80	5.1	10.5
12	36	18.88	−1.43	4.1	9.3
13	36	19.21	−1.83	4.8	10.3
14	36	19.57	−1.64	4.3	9.4
15	36	19.72	−2.06	5.1	10.4
16	36	19.90	−2.24	6.1	9.3
17	36	20.49	−1.80	4.9	9.6
18	36	20.72	−1.50	3.9	9.7
19	35	21.16	−1.44	3.8	9.7
20	36	21.21	−1.39	3.9	9.1
21	35	21.34	−1.72	4.4	9.6
22	36	21.67	−1.65	4.0	10.2
23	35	21.67	−1.84	4.6	9.9
24	36	22.07	−1.61	4.1	10.2
25	35	22.04	−1.75	4.4	10.4
26	36	22.10	−1.94	4.8	10.5
27	35	22.29	−1.71	4.2	10.3
28	36	22.54	−1.04	2.8	10.0
29	35	22.39	−1.45	3.5	10.3
30	34	22.91	−1.01	2.4	9.6
32	36	23.07	−1.11	2.6	10.8
34	36	23.42	−0.76	1.8	10.8
36	36	23.63	−1.02	2.4	10.9
38	36	24.01	−0.79	1.6	12.2
40	36	24.38	−0.65	1.4	11.8
42	36	24.44	−0.63	1.3	12.4
44	34	24.62	−0.90	1.5	13.1
46	34	24.68	−1.00	1.7	13.2
48	34	24.74	−0.71	1.2	13.7
50	34	25.38	−0.12	0.2	13.3
52	34	25.71	−0.05	0.1	15.4
54	33	25.82	+0.04	0.1	15.2

TABLE III—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
56	33	26.06	+0.06	0.1	15.0
58	33	26.26	±0.00	0.0	15.9
60	31	26.65	+0.53	0.7	14.5
62	31	26.69	+0.29	0.4	16.0
64	31	26.47	−0.17	0.2	16.3
66	30	27.02	+0.37	0.4	16.8
68	29	27.29	+0.74	0.9	16.9
70	29	26.90	+0.77	0.9	17.0
72	29	26.95	+0.39	0.5	16.6
74	29	27.07	+0.31	0.4	16.8
76	27	26.87	−0.37	0.4	15.7
78	27	26.72	−0.67	0.8	13.9
80	27	26.85	−0.79	0.9	16.2
82	27	27.15	−0.24	0.3	17.6
84	26	27.08	−0.31	0.3	17.6
86	25	27.34	−0.55	0.6	17.6
88	24	27.29	−0.48	0.5	14.4
90	23	26.93	−0.89	1.0	15.6
92	22	26.55	−1.43	1.6	11.6
94	22	26.52	−1.01	1.2	11.8
96	21	26.76	−1.27	1.4	13.2
98	20	26.58	−1.31	1.2	13.0
100	20	26.03	−1.39	1.3	13.7
102	20	25.88	−1.74	1.6	12.4
104	20	26.18	−0.88	0.9	12.3
106	17	24.97	−1.60	1.7	9.0
108	17	24.88	−1.94	1.8	10.5
110	17	24.82	−1.48	1.3	11.3
112	15	25.27	−1.43	1.4	8.9
114	15	25.30	+0.87	1.1	10.7
116	10	25.50	+0.93	1.0	12.3
118	8	24.50	+0.21	0.2	14.3
120	6	25.08	+0.94	0.8	13.9
122	6	24.75	−0.18	0.1	16.0
124	5	22.90	−2.03	1.8	14.4
126	5	22.50	−2.00	1.7	13.8
128	5	22.50	−3.30	2.7	15.8

TABLE IV.

Tethelin-Fed Males. Continuous Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	7	13.36	+0.98	—	—
5	20	13.05	+0.60	1.3	21.8
6	24	14.69	−0.89	1.9	18.2
7	24	16.54	−1.54	3.4	14.6
8	24	18.21	−1.15	2.6	12.0
9	24	18.98	−1.65	3.8	10.2
10	24	19.50	−1.69	3.6	10.8
11	24	19.92	−1.89	4.4	10.9
12	24	20.56	−2.09	4.8	10.6
13	24	20.88	−2.43	5.1	10.5
14	24	20.88	−3.08	6.2	11.2
15	24	21.21	−3.07	7.3	9.1
16	24	21.90	−2.85	6.5	8.4
17	24	22.65	−2.56	6.0	8.0
18	24	23.21	−2.40	5.7	7.3
19	24	23.79	−2.02	4.7	7.2
20	24	24.35	−1.75	4.6	6.3
21	24	24.52	−1.76	4.4	6.2
22	23	24.87	−1.19	3.1	7.6
23	23	24.61	−1.73	4.5	6.4
24	23	24.91	−1.91	4.7	7.7
25	23	25.33	−1.72	4.1	6.7
26	23	25.30	−1.64	3.6	8.1
27	23	25.59	−0.96	2.1	8.0
28	23	25.46	−1.73	3.8	7.4
29	23	25.63	−1.45	3.2	7.9
30	22	25.70	−1.53	3.7	7.2
32	23	26.00	−1.61	3.9	9.6
34	23	26.22	−1.49	3.2	8.8
36	23	26.02	−1.93	4.5	6.9
38	23	26.24	−1.96	4.4	7.4
40	23	26.43	−1.84	4.5	5.4
42	23	26.54	−1.74	3.9	5.8
44	23	26.50	−2.12	4.3	6.9
46	23	26.74	−1.81	3.7	7.0
48	23	26.78	−1.44	3.7	6.9
50	23	26.52	−2.36	4.8	7.8
52	23	26.98	−1.70	3.6	7.2
54	23	27.63	−1.04	2.1	7.0
56	23	27.72	−1.60	3.1	7.7
58	23	27.33	−1.77	3.8	6.7
60	23	26.93	−2.15	4.3	7.3

TABLE IV—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
62	23	27.46	−1.95	3.9	7.0
64	23	27.61	−1.92	3.8	7.2
66	23	27.22	−2.48	4.8	7.9
68	23	28.07	−1.73	3.4	7.9
70	23	28.11	−1.59	3.1	7.8
72	23	28.07	−1.63	3.1	7.4
74	23	27.96	−1.80	3.4	8.4
76	23	28.52	−1.46	3.0	7.7
78	23	28.17	−1.99	3.7	8.2
80	22	28.61	−1.34	2.3	9.2
82	21	28.50	−1.81	3.3	7.7
84	21	28.12	−1.76	3.0	7.9
86	21	28.38	−1.72	2.7	8.8
88	21	28.21	−1.98	3.1	8.4
90	19	29.32	−0.81	1.3	7.9
92	19	28.82	−1.64	2.6	8.4
94	19	28.89	−1.24	2.1	8.2
96	18	28.86	−0.81	1.4	8.7
98	18	28.83	−0.53	0.8	10.1
100	18	28.78	−0.76	1.4	8.2
102	18	28.58	−0.90	1.5	8.2
104	18	29.00	−0.61	1.0	8.5
106	18	28.78	−0.25	0.4	9.2
108	18	28.83	+0.27	0.5	7.9
110	18	28.72	+0.47	0.7	7.9
112	18	28.75	+0.45	0.7	7.8
114	18	28.72	+0.56	0.9	7.3
116	15	28.07	−0.12	0.2	6.7
118	13	28.08	−0.21	0.3	7.7
120	13	27.62	+0.37	0.5	7.3
122	13	27.73	+0.65	0.9	8.4
124	12	27.96	+0.64	0.9	7.6
126	12	27.75	+1.02	1.7	7.1
128	10	27.05	−0.50	0.8	3.8
130	9	26.89	−1.22	1.7	2.6
132	9	26.72	−1.28	1.3	3.9
134	7	26.57	−1.43	—	5.1
136	6	26.58	−0.92	—	5.7
138	5	26.50	+1.00	—	—
140	4	27.13	+1.13	—	—
142	3	26.67	—	—	—
144	2	26.00	—	—	—

TABLE V.

Tethelin-Fed Females. Discontinuous Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	12	10.96	+0.57	0.9	27.6
5	16	13.44	+1.63	3.4	19.6
6	16	14.94	+0.82	1.5	19.4
7	16	15.78	−0.99	1.9	16.1
8	16	16.19	−1.80	4.1	12.6
9	16	16.63	−2.15	4.8	12.4
10	16	17.03	−2.35	4.8	13.2
11	16	17.53	−2.51	4.6	15.7
12	16	17.66	−2.65	4.6	16.6
13	16	18.97	−2.07	3.8	14.1
14	16	19.72	−1.49	2.9	11.9
15	16	20.53	−1.25	2.4	11.4
16	16	20.19	−1.95	4.0	11.2
17	16	20.59	−1.70	3.4	11.5
18	16	20.31	−1.91	4.0	10.7
19	16	20.59	−2.01	3.9	11.9
20	16	20.59	−2.01	4.0	12.1
21	16	21.09	−1.97	3.9	11.2
22	16	21.13	−2.19	4.2	11.1
23	16	21.47	−2.04	3.8	12.2
24	16	21.78	−1.90	3.4	12.7
25	16	21.75	−2.04	3.6	12.8
26	16	22.00	−2.04	3.4	13.5
27	16	21.84	−2.16	3.7	13.6
28	16	22.22	−1.36	2.3	14.2
29	14	22.36	−1.48	2.3	14.1
30	16	22.38	−1.54	2.3	15.4
32	16	22.59	−1.79	2.4	15.4
34	16	23.06	−1.12	1.6	15.7
36	16	22.88	−1.77	2.6	15.7
38	16	23.44	−1.36	1.9	15.6
40	16	23.53	−1.50	2.0	16.9
42	16	23.53	−1.54	1.9	17.9
44	16	23.95	−1.58	1.8	18.8
46	16	23.59	−2.09	2.5	17.8
48	16	23.50	−1.95	2.4	17.1
50	16	24.09	−1.41	1.7	17.6
52	16	24.41	−1.35	1.6	16.6
54	16	24.41	−1.37	1.6	17.2
56	16	24.56	−1.44	1.6	17.1
58	16	24.38	−1.88	2.1	16.7
60	16	24.31	−1.81	2.0	16.8
62	16	24.28	−2.12	2.4	16.8

TABLE V—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
64	16	24.34	−2.30	2.5	16.7
66	16	24.16	−2.29	2.5	16.1
68	16	24.06	−2.49	2.9	14.7
70	16	24.06	−2.07	2.3	15.4
72	15	25.07	−1.49	1.6	16.3
74	15	24.70	−2.06	2.2	15.5
76	15	24.90	−2.34	2.5	15.7
78	15	24.73	−2.66	2.8	15.5
80	15	24.77	−2.87	2.9	16.4
82	15	23.93	−3.46	3.6	16.0
84	14	24.21	−3.54	3.6	16.6
86	13	24.73	−3.16	3.1	16.1
88	13	24.85	−2.92	2.6	17.9
90	13	24.54	−3.28	3.2	16.8
92	11	24.91	−3.07	2.8	15.1
94	11	25.41	−2.12	2.1	12.5
96	10	24.85	−3.18	3.2	12.6
98	8	24.56	−3.33	2.7	14.3
100	8	24.25	−3.17	2.5	14.2
102	8	24.56	−3.06	2.4	15.0
104	8	24.19	−2.87	2.5	13.1
106	8	24.00	−2.57	2.3	12.0
108	8	24.06	−2.76	2.2	13.4
110	8	23.44	−2.86	2.4	10.8
112	8	23.13	−3.57	3.3	10.3
114	8	23.31	−1.12	1.2	13.0
116	7	22.79	−1.78	1.9	12.0
118	7	23.36	−0.93	0.9	12.2
120	6	23.00	−1.14	1.2	16.1
122	6	23.08	−1.85	1.8	12.8
124	6	23.17	−1.76	1.8	12.5
126	5	23.80	−0.70	0.6	13.7
128	5	22.60	−3.20	3.0	12.8
130	5	22.70	−3.10	—	13.4
132	5	22.90	−2.20	—	13.3
134	5	22.50	−3.00	—	11.1
136	4	22.13	−1.87	—	—
138	4	21.75	−2.75	—	—
140	4	21.88	−2.75	—	—
142	4	21.50	−3.38	—	—
144	3	23.33	−0.67	—	—
146	2	22.50	−2.00	—	—
148	2	22.50	−1.50	—	—

TABLE VI.

Lecithin-Fed Males.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	18	10.86	−1.52	3.2	23.1
5	36	11.53	−0.92	2.6	23.2
6	36	13.11	−2.47	5.9	21.7
7	36	15.07	−3.01	6.7	19.4
8	36	16.56	−2.80	5.9	18.5
9	36	17.69	−2.94	6.2	15.8
10	36	19.13	−2.06	4.4	13.7
11	36	20.06	−1.75	4.4	11.4
12	36	20.50	−2.15	5.5	10.2
13	36	21.10	−2.21	5.0	9.8
14	36	21.49	−2.47	5.6	9.4
15	36	22.17	−2.11	5.2	9.9
16	36	22.22	−2.53	6.2	8.9
17	36	22.60	−2.61	6.4	8.5
18	36	23.25	−2.36	5.6	8.7
19	36	23.72	−2.09	5.1	7.5
20	36	24.04	−2.06	5.4	8.0
21	36	24.17	−2.11	5.3	8.2
22	36	24.86	−1.20	3.5	7.6
23	36	24.57	−1.77	4.8	7.9
24	36	24.43	−2.39	6.1	8.7
25	34	24.50	−2.55	6.1	8.6
26	36	25.10	−1.84	4.4	8.4
27	34	24.97	−1.58	3.8	7.4
28	35	25.26	−1.93	4.4	8.8
29	33	25.27	−1.81	4.1	8.8
30	34	25.74	−1.49	3.7	8.8
32	36	26.04	−1.57	3.8	9.6
34	36	26.32	−1.39	3.2	8.5
36	35	26.86	−1.09	2.5	8.8
38	35	26.84	−1.36	3.0	8.8
40	33	27.02	−1.25	2.6	8.3
42	33	27.23	−1.05	2.1	9.5
44	33	27.15	−1.47	2.8	10.0
46	33	27.17	−1.38	2.7	10.1
48	32	27.47	−0.75	1.6	9.1
50	31	27.44	−1.44	2.9	9.7
52	31	27.76	−0.92	1.9	9.3
54	30	28.07	−0.60	1.2	8.7
56	30	27.82	−1.50	2.8	9.0

TABLE VI—*Continued.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
58	30	27.93	−1.17	2.5	7.8
60	28	27.75	−1.33	2.6	8.1
62	28	27.50	−1.91	3.9	7.5
64	28	27.93	−1.60	3.3	7.6
66	28	27.86	−1.84	3.8	7.4
68	27	27.44	−2.36	4.8	7.3
70	26	27.81	−1.89	3.9	7.8
72	25	27.66	−2.04	4.1	6.4
74	25	27.60	−2.16	4.1	8.1
76	24	27.50	−2.48	5.2	7.6
78	22	27.20	−2.96	6.4	5.1
80	22	27.56	−2.39	4.9	6.5
82	22	27.16	−3.15	5.8	7.5
84	22	26.98	−2.90	5.4	6.0
86	20	27.00	−3.10	5.2	6.7
88	19	27.21	−2.98	5.0	6.0
90	18	27.39	−2.74	4.8	6.4
92	18	27.36	−3.10	5.6	5.2
94	17	26.91	−3.22	5.9	6.6
96	16	26.63	−3.04	5.7	6.8
98	16	26.93	−2.43	6.1	6.5
100	16	26.31	−3.23	6.2	7.6
102	16	26.81	−2.67	4.8	6.3
104	16	26.38	−3.23	5.9	6.1
106	16	26.28	−2.75	4.7	6.3
108	15	26.99	−1.57	2.9	6.3
110	13	25.65	−2.60	4.2	6.3
112	13	26.42	−1.88	2.9	6.6
114	13	26.00	−2.16	3.5	6.1
116	11	26.27	−1.92	2.4	8.1
118	11	25.32	−2.97	4.2	5.6
120	10	25.50	−1.75	2.3	6.0
122	9	24.89	−2.19	3.4	5.9
124	9	24.83	−2.49	3.9	4.8
126	9	24.67	−2.06	3.8	5.0
128	9	24.39	−3.16	4.6	5.6
130	9	24.28	−3.83	5.0	6.0
132	9	24.39	−3.61	3.5	7.0
134	9	23.56	−4.44	—	5.9
136	9	23.50	−4.00	—	7.1
138	6	24.17	−1.33	—	4.1

TABLE VI—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
140	6	24.08	−1.92	—	4.6
142	5	25.00	—	—	4.6
144	4	24.50	—	—	1.5
146	4	24.00	—	—	2.5
148	3	24.17	—	—	—
150	3	24.50	—	—	—
152	3	24.67	—	—	—
154	3	24.67	—	—	—
156	3	20.83	—	—	—
158	3	21.67	—	—	—
160	2	20.25	—	—	—
162	2	21.50	—	—	—
164	1	21.50	—	—	—
166	1	21.00	—	—	—

TABLE VII.

Lecithin-Fed Females.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
5	31	11.42	−0.39	1.4	15.8
6	36	13.35	−0.77	2.7	12.7
7	36	15.04	−1.73	5.4	9.7
8	36	16.47	−1.52	4.8	8.8
9	36	17.00	−1.78	5.6	8.6
10	36	17.32	−2.06	5.6	9.6
11	36	18.08	−1.96	5.9	8.2
12	36	18.67	−1.64	4.8	8.6
13	35	18.71	−2.33	6.7	8.2
14	35	19.26	−1.95	5.6	7.6
15	35	19.72	−2.06	5.4	8.9
16	35	20.03	−2.11	5.9	8.4
17	35	20.26	−2.03	5.7	8.5
18	35	20.50	−1.72	4.6	9.0
19	35	20.77	−1.83	4.9	8.6
20	35	20.64	−1.96	5.8	8.5
21	35	21.03	−2.03	5.3	8.8
22	35	21.13	−2.19	5.6	9.1
23	35	21.31	−2.20	5.6	9.0
24	35	21.27	−2.41	6.3	9.4
25	35	21.34	−2.45	6.3	9.4
26	35	21.66	−2.38	6.3	8.9
27	35	21.61	−2.39	6.0	9.8
28	33	21.92	−1.66	4.5	9.8
29	35	22.06	−1.78	4.3	10.2
30	29	22.34	−1.58	3.6	10.6
32	34	22.29	−1.89	4.4	11.2
34	33	22.56	−1.62	3.7	10.9
36	33	22.88	−1.77	4.1	10.8
38	33	22.82	−1.98	4.2	11.4
40	33	22.97	−2.06	4.6	11.2
42	33	23.00	−2.07	4.1	12.1
44	33	23.29	−2.23	3.9	12.9
46	32	23.20	−2.48	4.4	13.6
48	32	24.02	−1.43	2.4	14.2
50	31	24.08	−1.42	2.4	14.4
52	31	24.60	−1.16	1.8	14.0
54	29	24.55	−1.23	1.8	14.7
56	29	24.28	−1.72	2.5	14.1
58	29	24.69	−1.57	2.3	13.5
60	28	24.09	−2.03	2.9	13.1
62	28	23.73	−2.67	3.9	12.4

TABLE VII—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
64	28	24.34	−2.30	3.2	12.9
66	28	24.82	−1.63	2.1	13.1
68	28	23.86	−2.69	3.5	14.4
70	27	24.61	−1.52	1.9	14.4
72	25	23.26	−3.30	4.2	15.0
74	22	24.41	−2.35	2.9	14.0
76	22	24.77	−2.47	3.0	14.5
78	22	24.93	−2.46	2.9	14.3
80	22	24.73	−2.91	3.4	14.4
82	22	24.57	−2.82	3.4	14.4
84	21	24.19	−3.56	4.3	14.5
86	21	24.31	−3.58	3.9	15.8
88	21	24.52	−3.25	3.5	15.7
90	20	24.55	−3.27	3.5	17.6
92	19	24.79	−3.19	3.1	17.8
94	19	24.32	−3.21	3.1	18.4
96	19	24.05	−3.98	3.8	18.8
98	18	24.53	−3.36	3.0	17.3
100	16	23.88	−3.54	3.1	16.6
102	16	23.75	−3.87	3.5	14.9
104	13	22.96	−4.10	3.9	13.8
106	13	23.38	−3.19	3.0	14.8
108	12	22.75	−4.07	3.6	12.4
110	11	22.50	−3.80	3.2	13.3
112	9	22.94	−3.76	3.5	10.5
114	8	22.75	−1.68	1.9	10.9
116	8	22.88	−1.69	1.8	12.8
118	8	22.69	−1.60	1.8	11.3
120	8	22.69	−1.45	1.7	11.1
122	7	21.93	−3.00	3.3	10.0
124	6	21.00	−3.93	4.6	10.6
126	5	21.40	−3.10	3.1	12.0
128	5	21.30	−4.50	4.5	12.3
130	5	21.20	−4.60	4.5	12.9
132	5	21.20	−3.90	—	12.6
134	4	20.50	−5.00	—	10.2
136	4	20.25	−3.75	—	11.2
138	3	21.50	−3.00	—	—
140	2	22.00	−2.63	—	—
142	2	21.75	−3.13	—	—
144	2	21.50	−3.00	—	—
146	1	19.00	−5.50	—	—

TABLE VIII.

Cholesterol-Fed Males.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
5	22	12.84	+0.39	1.0	18.1
6	33	14.06	−1.52	3.6	19.3
7	34	15.85	−2.23	5.2	16.2
8	34	16.88	−2.48	5.6	15.5
9	34	16.79	−3.84	7.8	17.9
10	34	17.41	−3.78	7.3	18.4
11	34	17.85	−3.96	8.2	18.2
12	34	18.96	−3.69	7.7	16.1
13	34	20.46	−2.85	5.8	13.5
14	34	21.37	−2.59	5.3	12.4
15	34	21.88	−2.40	5.2	13.3
16	34	22.03	−2.72	5.7	12.9
17	32	22.20	−3.01	6.7	11.0
18	34	23.10	−2.51	5.3	11.5
19	32	22.55	−3.26	7.4	9.9
20	34	23.37	−2.73	6.2	11.0
21	30	23.47	−2.81	6.5	9.1
22	34	24.19	−1.87	4.7	10.5
23	30	24.02	−2.32	5.7	9.4
24	29	24.43	−2.39	5.3	10.3
25	29	24.24	−2.81	6.3	8.9
26	29	24.48	−2.46	5.3	10.5
27	29	24.16	−2.39	5.3	9.2
28	29	25.00	−2.19	4.6	10.4
29	29	24.59	−2.49	5.5	9.1
30	28	24.77	−2.46	5.1	11.2
32	34	25.34	−2.27	4.9	11.1
34	34	25.62	−2.09	4.2	11.3
36	34	25.66	−2.29	4.7	11.8
38	33	26.18	−2.02	4.3	9.9
40	27	26.06	−2.21	4.1	11.8
42	32	26.19	−2.09	3.9	11.5
44	32	26.27	−2.35	4.4	10.4
46	32	27.00	−1.55	2.9	11.7
48	32	26.84	−1.38	2.9	9.4
50	32	26.83	−2.05	4.2	9.3
52	32	26.78	−1.90	3.8	10.5
54	32	27.50	−1.17	2.2	9.9
56	32	26.98	−2.34	4.3	9.8

TABLE VIII—*Continued.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
58	32	26.78	−2.32	4.4	11.3
60	32	26.88	−2.20	4.0	10.8
62	32	27.06	−2.35	4.4	10.1
64	32	26.98	−2.55	4.8	10.2
66	32	26.95	−2.75	5.0	10.8
68	32	27.06	−2.74	5.1	10.9
70	31	27.23	−2.47	4.7	10.2
72	31	27.23	−2.47	4.3	10.9
74	31	27.31	−2.45	4.4	11.1
76	29	27.59	−2.39	4.5	10.8
78	29	26.93	−3.23	5.7	11.1
80	29	27.41	−2.54	4.6	10.3
82	28	27.71	−2.60	4.3	10.9
84	27	27.13	−2.75	4.3	11.8
86	27	27.28	−2.82	4.1	12.4
88	27	27.67	−2.52	3.7	11.3
90	27	27.31	−2.82	4.2	12.3
92	27	27.00	−3.46	5.2	12.1
94	26	26.79	−3.34	5.0	13.7
96	26	26.81	−2.86	4.5	13.0
98	25	26.46	−2.90	4.5	13.4
100	23	26.22	−3.32	5.4	14.4
102	22	26.37	−3.11	4.3	13.9
104	22	26.43	−3.18	4.5	13.6
106	21	26.43	−2.60	3.7	12.4
108	19	26.24	−2.32	3.5	12.0
110	19	26.05	−2.20	2.9	12.8
112	18	25.64	−2.66	3.3	14.0
114	18	25.78	−2.38	3.0	13.7
116	17	24.94	−3.25	3.7	13.8
118	15	24.40	−3.89	4.2	15.5
120	13	24.92	−2.33	2.5	15.4
122	10	25.40	−1.68	1.5	17.6
124	9	25.56	−1.76	1.8	17.3
126	8	23.94	−2.79	3.0	14.1
128	7	24.36	−3.19	3.0	14.0
130	5	25.50	−2.61	2.6	9.6
132	4	25.63	−2.37	1.6	12.4
134	4	24.38	−3.62	—	10.3
136	3	24.50	−3.00	—	—

TABLE VIII—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
138	2	24.25	−1.25	—	—
140	2	24.25	−1.75	—	—
142	2	24.00	—	—	—
144	2	24.25	—	—	—
146	2	24.25	—	—	—
148	2	22.50	—	—	—
150	2	22.75	—	—	—
152	2	22.25	—	—	—
154	1	24.00	—	—	—
156	1	23.00	—	—	—
158	1	22.00	—	—	—
160	1	22.00	—	—	—

TABLE IX.

Cholesterol-Fed Females.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
5	14	13.14	+1.33	2.5	21.5
6	35	13.10	−1.02	3.2	15.6
7	35	13.90	−2.87	7.5	16.5
8	35	14.90	−3.09	8.1	15.4
9	35	15.10	−3.68	8.8	17.8
10	35	15.54	−3.84	9.4	15.2
11	35	16.06	−3.98	9.3	18.1
12	35	17.10	−3.21	9.5	16.1
13	35	17.73	−3.31	9.2	16.9
14	35	18.46	−2.75	6.2	14.7
15	35	18.77	−3.01	6.8	13.7
16	35	19.21	−2.93	7.0	13.3
17	33	19.30	−2.99	7.3	12.8
18	34	19.66	−2.56	6.2	12.8
19	33	19.65	−2.95	7.0	12.3
20	34	20.12	−2.48	6.4	12.1
21	33	20.24	−2.82	6.4	12.9
22	34	20.35	−2.97	6.7	12.4
23	33	20.48	−3.03	6.8	12.4
24	33	20.80	−2.88	6.3	14.3
25	33	20.94	−2.85	6.1	14.5
26	33	21.24	−2.80	5.8	14.7
27	33	21.32	−2.68	5.5	14.8
28	33	21.56	−2.02	4.2	15.7
29	33	21.62	−2.22	4.4	15.2
30	34	22.07	−1.85	3.4	16.6
32	33	22.08	−2.10	3.8	17.3
34	32	22.28	−1.90	3.2	18.4
36	32	22.41	−2.24	3.7	19.9
38	32	22.89	−1.91	2.9	20.4
40	32	22.88	−2.14	3.2	21.0
42	31	22.90	−2.17	3.0	22.8
44	30	23.30	−2.22	2.8	23.1
46	29	24.14	−1.54	1.9	23.0
48	29	23.86	−1.59	1.9	23.4
50	28	23.86	−1.64	2.0	23.0
52	27	24.06	−1.70	2.0	22.4
54	27	24.61	−1.17	1.3	21.9
56	27	24.30	−1.70	1.9	23.1
58	26	24.13	−2.13	2.3	23.0
60	26	24.35	−1.77	2.0	21.1
62	25	24.66	−1.74	1.9	22.3

TABLE IX—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
wks.		*gm*			*per cent*
64	25	24.38	−2.26	2.4	21.7
66	25	24.28	−2.17	2.2	21.6
68	23	23.91	−2.64	3.3	15.5
70	23	23.87	−2.26	2.8	15.5
72	23	24.24	−2.32	2.7	16.9
74	23	24.04	−2.72	3.2	15.8
76	23	23.91	−3.33	3.9	16.8
78	23	23.96	−3.43	4.0	16.0
80	22	24.05	−3.59	3.9	17.9
82	20	24.15	−3.24	3.4	18.5
84	19	24.16	−3.59	3.7	19.1
86	18	24.06	−3.83	3.7	19.3
88	16	24.31	−3.46	3.4	16.5
90	16	24.38	−3.44	3.6	16.2
92	13	24.31	−3.67	3.3	17.4
94	13	24.50	−3.03	3.0	14.8
96	11	24.05	−3.98	3.5	17.1
98	10	22.55	−5.34	4.6	14.7
100	10	23.30	−4.12	3.3	15.9
102	10	23.00	−4.62	3.9	14.2
104	10	23.45	−3.61	3.3	13.3
106	10	23.25	−3.32	3.0	13.7
108	10	22.65	−4.17	3.6	12.9
110	9	22.22	−4.08	3.4	13.1
112	9	22.61	−4.09	3.6	13.0
114	9	22.44	−1.99	2.2	13.0
116	7	22.50	−2.07	2.3	12.2
118	7	21.29	−3.00	3.0	13.1
120	6	21.08	−3.06	3.3	11.9
122	6	21.50	−3.43	3.1	14.6
124	6	21.25	−3.68	3.9	12.3
126	6	21.58	−2.92	2.9	13.0
128	5	22.30	−3.50	3.7	10.9
130	5	22.00	−3.80	—	11.2
132	4	21.25	−3.85	—	6.4
134	4	21.13	−4.37	—	7.8
136	4	21.00	−3.00	—	8.4
138	4	20.38	−4.12	—	6.1
140	4	20.38	−4.25	—	5.6
142	4	20.63	−4.25	—	4.7
144	4	19.25	−5.25	—	7.1
146	3	19.50	−5.00	—	—
148	1	19.50	−4.50	—	—
150	1	16.50	−7.50	—	—

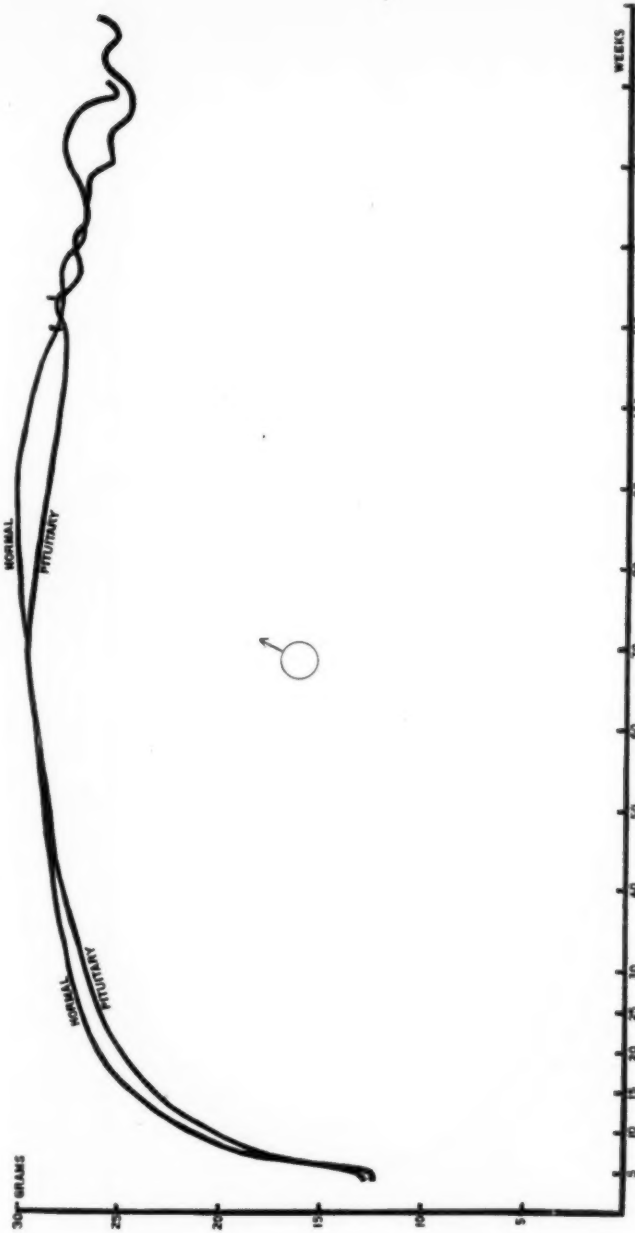

FIG. 1. Influence of pituitary (anterior lobe) tissue upon the growth of male white mice. Dosage one-twelfth of an ox gland per mouse per day. The vertical cross mark indicates average duration of life.

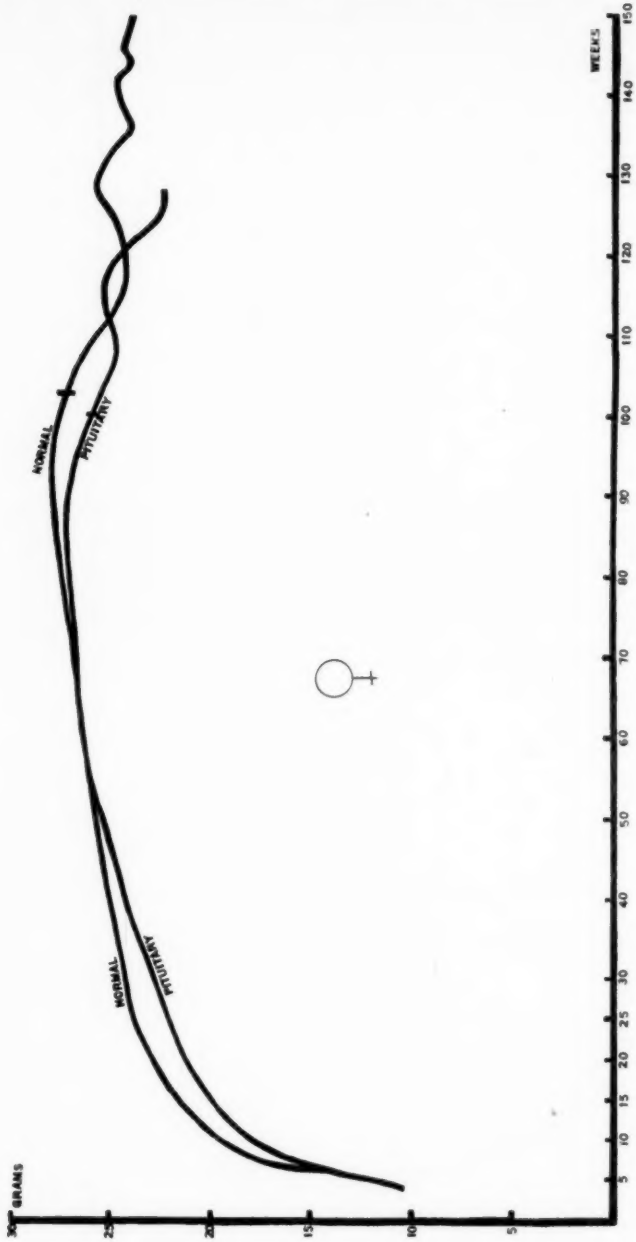

Fig. 2. Influence of pituitary (anterior lobe) tissue upon the growth of female white mice. Dosage one-twelfth of an ox gland per mouse per day. The vertical cross mark indicates average duration of life.

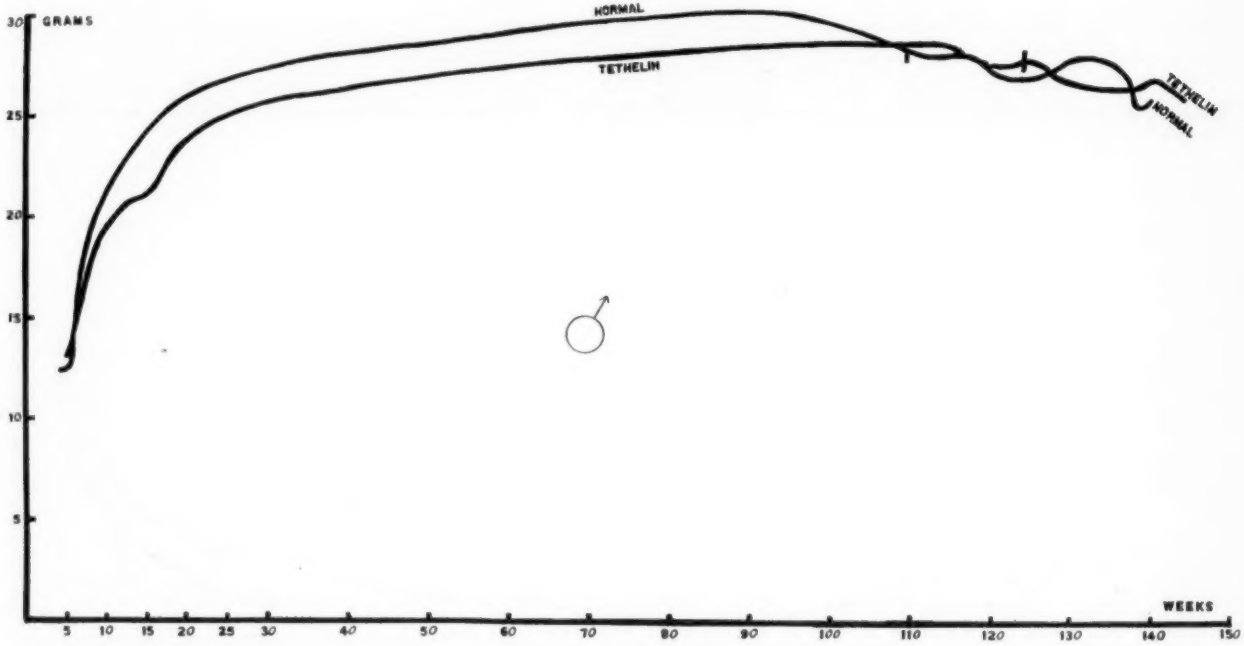

FIG. 3. Influence of tethelin upon the growth of male white mice. Dosage 4 mg. per day. The vertical cross mark indicates average duration of life.

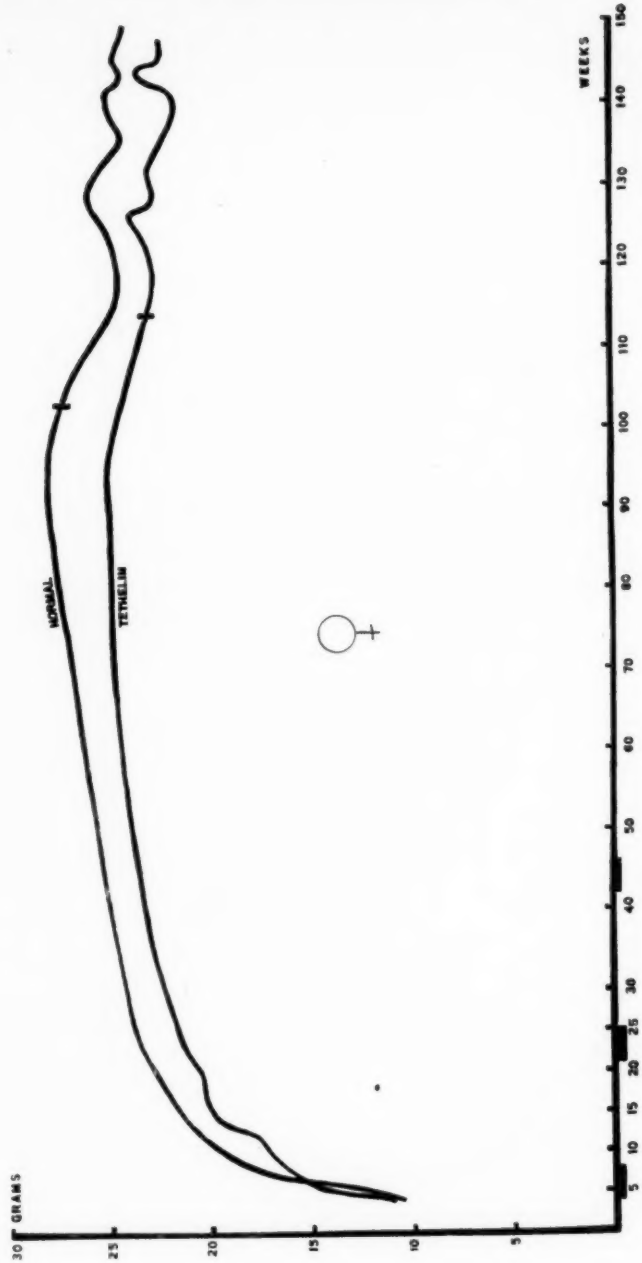

FIG. 4. Influence of tethelin upon the growth of female white mice. Administration of 4 mg. per day in three periods of 4 weeks each, indicated by the heavily shaded portions of the base line. The vertical cross mark indicates average duration of life.

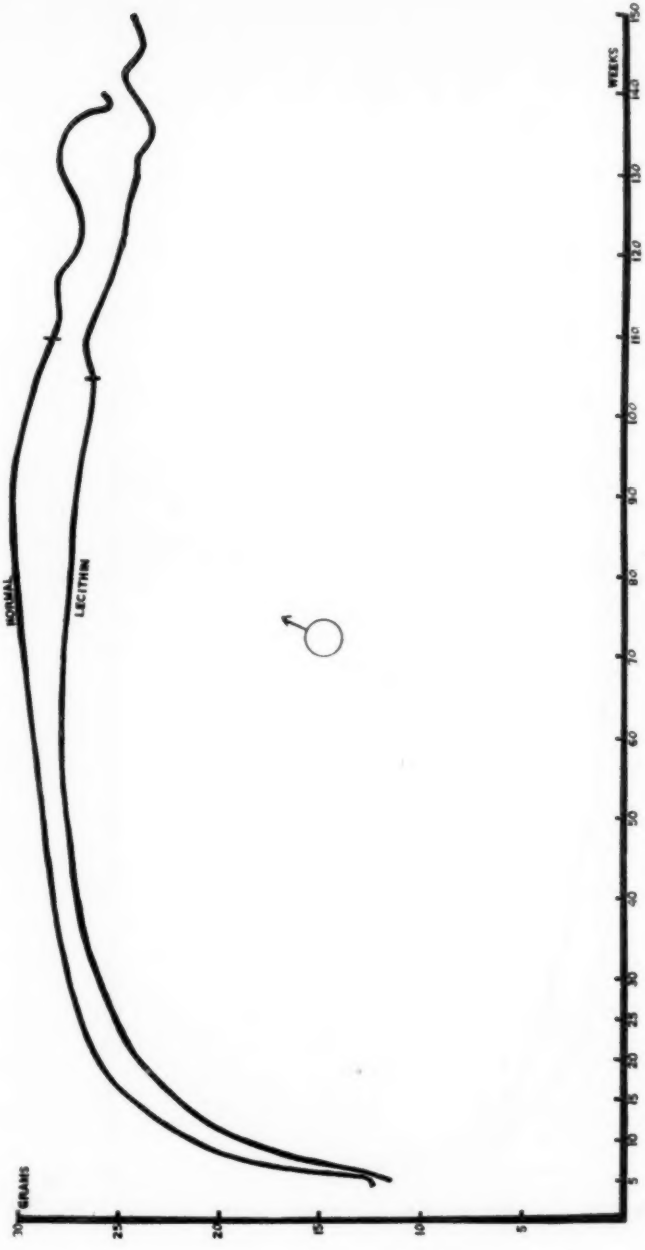

FIG. 5. Influence of lecithin upon the growth of male white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

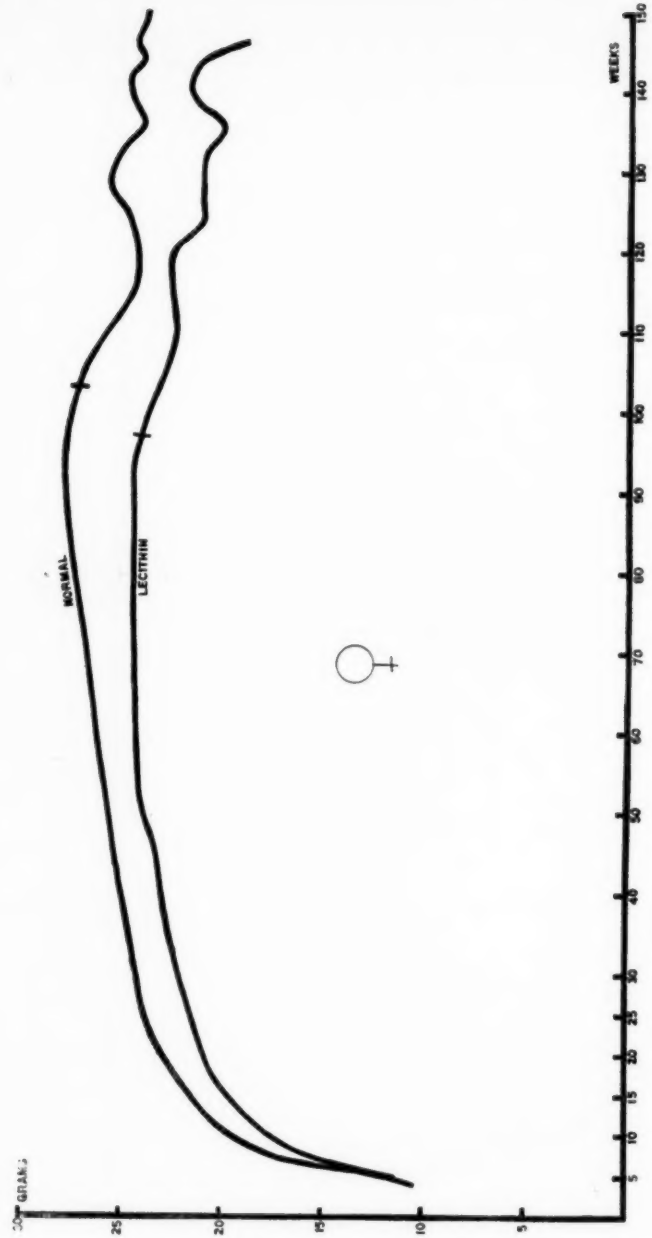

FIG. 6. Influence of lecithin upon the growth of female white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

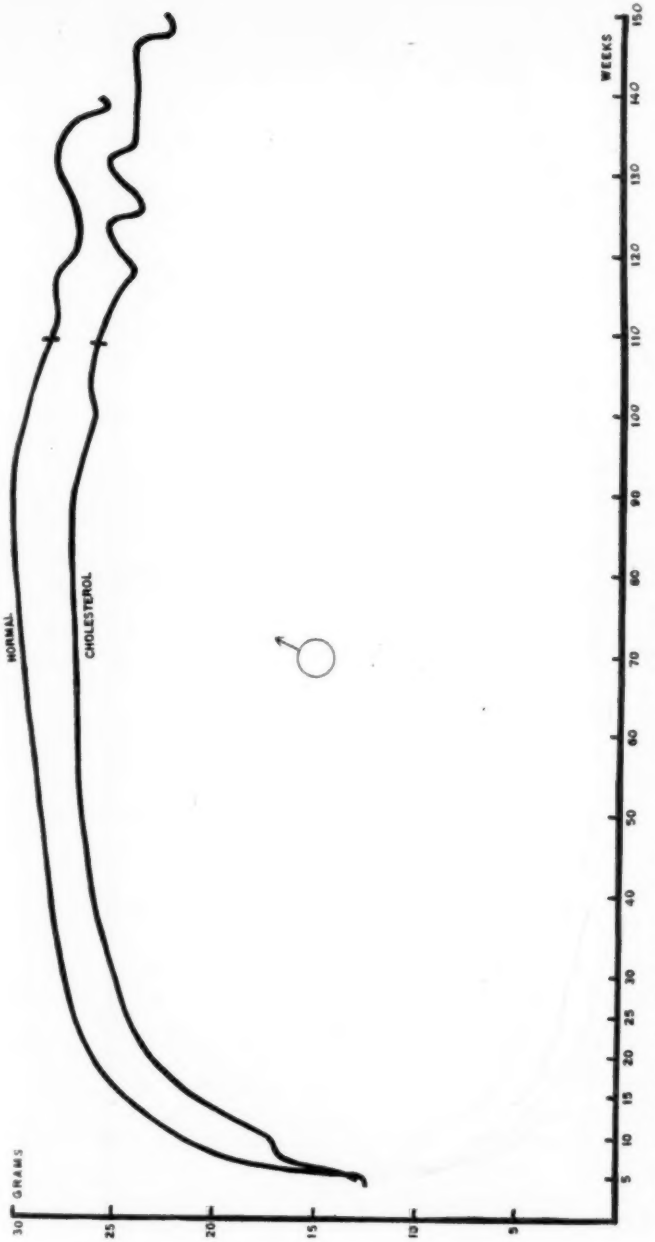

FIG. 7. Influence of cholesterol upon the growth of male white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

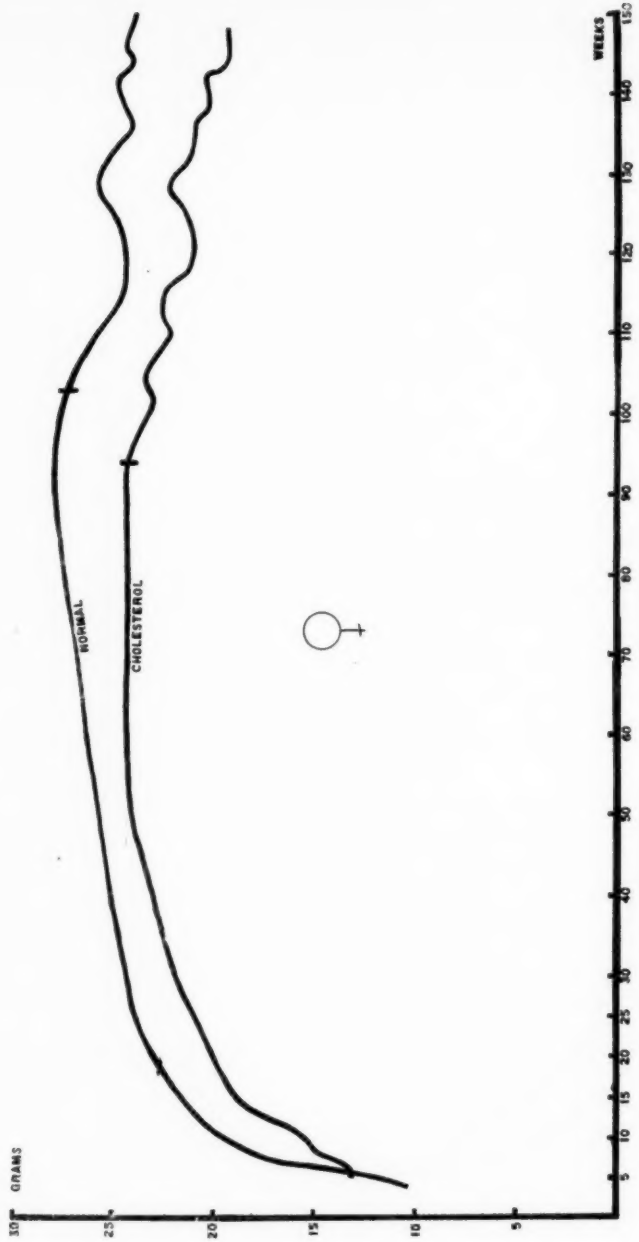

FIG. 8. Influence of cholesterol upon the growth of female white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

EXPERIMENTAL STUDIES ON GROWTH.

XII. THE INFLUENCE OF PITUITARY GLAND (ANTERIOR LOBE) TISSUE, TETHELIN, EGG LECITHIN, AND CHOLESTEROL UPON THE DURATION OF LIFE OF THE WHITE MOUSE.

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Methods of Estimation.

Previous estimates of the duration of life of animals, such as those cited by Friedenthal,[1] are for the most part based upon single or relatively few observations, and the duration of life is frequently estimated to be that of the longest lived animal observed. So far as we are aware, no statistical estimation of the life duration of animals has ever been attempted.

According to Mitchell[2] mice of various species in captivity live for from 16 to 20 months, being presumably grown animals when first introduced to captivity. He quotes Metchnikoff, without detailing the evidence, to the effect that mice may attain the age of 6 years, an estimate which is certainly exaggerated. According to Wood,[3] the "expectation of life" for a mouse is $2\frac{1}{2}$ years, but he does not enumerate the data upon which he bases this estimate, which, as we shall see, is somewhat in excess of the correct figure.

All the animals used in the experiments reported in Papers I to VI and Paper XII of this series were retained and the dietary administrations continued,[4] under exactly the same conditions as

[1] Friedenthal, H., *Zentr. Physiol.*, 1911, xxiv, 321.

[2] Mitchell, P. C., *Proc. Zool. Soc. London*, 1911, pt. 2, 469.

[3] Wood, F. C., *J. Am. Med. Assn.*, 1916, lxvi, 94.

[4] The administration of cholesterol to the cholesterol-fed animals was, however, discontinued at the end of the 2nd year owing to our inability to procure cholesterol on the market, and lack of time and facilities for producing the large quantities of cholesterol required.

those reported in Paper I,[5] throughout their lives. The dosage of pituitary tissue was one-twelfth of an ox gland (anterior lobe) per mouse per day; that of tethelin was 4 mg. per day, and that of lecithin and of cholesterol was 40 mg. per day.

When possible death was allowed to occur naturally; but occasionally, when it appeared that valuable material for postmortem examination might otherwise be lost natural death was anticipated by a few hours or at most a few days, by etherization.

In estimating the part played by senescence in determining the duration of life, deaths which are essentially accidental must be excluded. Such deaths include those due to epidemic infections. The technique adopted in these experiments practically excluded epidemic infections from among the causes of mortality. In only two instances were epidemic infections encountered; namely, when six normal females in one nest developed pneumonia following one case which was not isolated promptly, and when a whole cage of animals (twenty-four) developed a subacute infection of the lungs and nasal epithelium. In the former instance the animals were destroyed and their deaths regarded as accidental and not included in the estimate of the duration of life. In the second instance the whole experiment affected was terminated and its results were rejected.

The majority of accidental deaths occurred in the first few months of life, and in the first few weeks after the transfer of the animals from the breeding cages to the experimental cages, sporadic cases of β-paratyphoid frequently developed. Such animals were killed, and their nests and cages sterilized. Fatal injuries due to fighting also occurred among the young males. For these reasons all deaths prior to 210 days, when the animals may be regarded as adult, are regarded as accidental and rejected in the estimation of the average duration of life.

On the other hand, *sporadic* infections occurring subsequently to 210 days are not regarded as accidental, but are considered to have been determined by the condition and general resistance of the animals, so that death due to local or generalized infections occurring in single animals was probably occasioned not by exceptional exposure to infection, but by lack of resistance to for-

[5] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347.

merly acquired and chronic latent infections, resulting from impairment of the vitality of the tissues by senescence. It was frequently observed, particularly in the case of infections of the genitourinary organs in males, that injuries from fighting would be received early in life and would heal completely, to all external appearance. Late in life, usually at an advanced age (*i.e.*, exceeding the mean duration of life), abscesses would form at the spot previously injured. It was inferred that a localized infection had been present from the date of injury, but that the general resistance of the animals had been sufficiently strong to prevent the infection from making headway until senescence and the consequent impairment of resistance gave it an opportunity to proceed and to terminate the life of the animal.

Results.

The results obtained are summarized in Table I and are shown in detail in Tables II to XI and Figs. 1 to 10. In the case of the pituitary tissue and of the lecithin and cholesterol administration the deviations of the mean duration of life from the normal lie within or almost within the probable error of estimation (about 1 month).

In the two groups of animals which received tethelin, however, the duration of life was greatly extended, exceeding the normal by 99 days, or 13 per cent of the normal duration of life in the males which received tethelin continuously, and by 81 days, or 11 per cent of the normal duration of life in the females which received tethelin intermittently in three periods of 1 month each prior to the 30th week.

The experiments on both sexes confirm each other. Since the determinations were carried out in both sexes in the same manner and yet independently, the chance of both observed deviations from normal being accidental in origin may be estimated by multiplying the separate chances of their being accidental. In this way we find that the deviations from the normal duration of life shown by the pituitary-fed and cholesterol-fed animals were more probably accidental than not, and that those displayed by the lecithin-fed animals were almost as probably accidental as not (1 to 1.4). In the case of the tethelin-fed groups, on the

contrary, there is only one chance in 150 that the deviations from the normal duration of life can have been accidental. The correlation of the magnitude of the deviation with the dosage of tethelin administered to the males and to the females respectively adds strong confirmation to this conclusion.

Since none of the ten groups of animals investigated, with the exception of the two tethelin-fed groups, shows any deviation

TABLE I.

Summary of Tables II to XII.

Class of animals.	Average duration of life.	Deviation from normal.	Ratio of deviation to probable error.	Chance that observed deviation was accidental.
		Males.		
	days	*days*		
Normal	767	—	—	—
Pituitary	792	+25	0.76	1 to 0.64
Cholesterol	764	− 3	0.10	1 " 0.06
Lecithin	731	−36	0.94	1 " 0.90
Tethelin	866	+99	3.00	1 " 22.25
		Females.		
Normal	719	—	—	—
Pituitary	704	−15	0.48	1 to 0.34
Cholesterol	658	−61	1.72	1 " 3.06
Lecithin	677	−42	1.27	1 " 1.55
Tethelin	800	+81	2.25	1 " 6.75

Chances that the results obtained with both sexes were accidental.

Pituitary	1 to 0.2
Cholesterol	1 " 0.2
Lecithin	1 " 1.4
Tethelin	1 " 150.2

from the normal duration of life which may not be regarded as lying within the experimental error of the estimation, we may regard the normal, pituitary-fed, lecithin-fed, and cholesterol-fed animals as forming collectively a series of control animals and estimate from this increased number the normal duration of life with a probable error reduced to approximately one-half. In this way we find that the prolongation of life of the tethelin-fed

males beyond the average of all other classes of males was 103 days, while that of the tethelin-fed females was 108 days, and the chance that both these deviations from the norm thus established were accidental is one in 11,000.

In every group of animals there is a tendency, more or less marked, to bimodality of the frequency curve of death, or curve of the rate of death at varying ages. This tendency is, however, exaggerated in both the groups of tethelin-fed animals, so that two periods of mortality are separated by a period during which the death rate sank to zero. The smaller proportion of the animals died relatively early in life; the larger proportion died in the later period of mortality. The prolongation of the mean duration of life is attributable to the remarkable longevity of the latter group.

The *median* duration of life, or age at which 50 per cent of the animals had expired, was determined graphically from the curve of total deaths by determining the point of intersection with the straight line $y = 50$ per cent. In every case except the cholesterol-fed males and the pituitary-fed females the median duration of life is less than the average duration of life, implying that the animals which survive beyond the median duration of life are to a slight extent selected animals, not differing fortuitously, but to some extent in kind from those which die earlier. This tendency towards asymmetry of the distribution of deaths is slight, the deviation of the median from the mean only definitely exceeding the probable error of the estimation in two instances (cholesterol-fed females and lecithin-fed males). In the case of the tethelin-fed females the median duration of life was indeterminate, because it fell within the period of zero mortality when a single death would have affected the estimate by no less than 100 days.

Although the lecithin-fed animals displayed a diminished rather than an enhanced viability, yet a small group of males attained extraordinary longevity, 9 per cent surviving to over 1,100 days, as compared with 0 per cent of normals, 3 per cent of each of the pituitary- and cholesterol-fed groups, and 4 per cent of the tethelin-fed group. Had we relied solely upon the longevity of the longest lived individual in estimating the duration of life, therefore, we should have been led to select lecithin as the dietary addition most favorable to prolongation of existence,

whereas, the statistical investigation reveals that it is actually unfavorable. The females did not plainly exhibit any similar tendency, and we remain uncertain whether the remarkable longevity of the small group of males referred to was fortuitous in origin, or attributable to the lecithin or to other substances contained in the crude preparation of lecithin employed.

TABLE II.

Mortality of Normal Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
200–300	2	6.0	6.0	241
300–400	0	0.0	6.0	—
400–500	0	0.0	6.0	—
500–600	4	12.5	18.5	555
600–700	4	12.5	31.0	652
700–800	6	19.0	50.0	731
800–900	5	16.0	66.0	847
900–1,000	9	28.0	94.0	952
1,000–1,100	2	6.0	100.0	1,021

Total No. of animals	32
Average duration of life	767 days
Standard deviation of duration of life	200 "
Probable error of average	±24 "
Variability of the duration of life	26 per cent
Median duration of life (50 per cent dead)	731 days
Probable error of median	±30 "

TABLE III.

Mortality of Normal Females.

Age-group	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
200–300	1	3	3	293
300–400	2	6	9	348
400–500	2	6	15	495
500–600	5	16	31	545
600–700	3	9	40	684
700–800	9	28	68	760
800–900	5	16	84	845
900–1,000	1	3	87	967
1,000–1,100	4	13	100	1,050

Total No. of animals	32
Average duration of life	719 days
Standard deviation of duration of life	204 "
Probable error of average	±24 "
Variability of the duration of life	28 per cent
Median duration of life (50 per cent dead)	710 days
Probable error of median	±30 "

TABLE IV.

Mortality of Pituitary-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
300–400	1	3	3	330
400–500	1	3	6	484
500–600	2	6	12	523
600–700	3	10	22	636
700–800	8	26	48	745
800–900	9	29	77	853
900–1,000	3	10	87	917
1,000–1,100	3	10	97	1,033
1,100–1,200	1	3	100	1,181

Total No. of animals	31
Average duration of life	792 days
Standard deviation of duration of life	180 "
Probable error of average	±22 "
Variability of the duration of life	23 per cent
Median duration of life (50 per cent dead)	750 days
Probable error of median	±27 "

TABLE V.

Mortality of Pituitary-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
300–400	2	6	6	388
400–500	5	14	19	446
500–600	3	9	28	563
600–700	3	9	37	634
700–800	7	20	57	751
800–900	12	34	91	843
900–1,000	3	9	100	926

Total No. of animals	35
Average duration of life	704 days
Standard deviation of duration of life	177 "
Probable error of average	±20 "
Variability of the duration of life	25 per cent
Median duration of life (50 per cent dead)	715 days
Probable error of median	±25 "

TABLE VI.

Mortality of Tethelin-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
500–600	2	9	9	568
600–700	3	13	22	644
700–800	0	0	22	—
800–900	7	30	52	842
900–1,000	5	22	74	940
1,000–1,100	5	22	96	1,025
1,100–1,200	1	4	100	1,124

Total No. of animals	23
Average duration of life	866 days
Standard deviation of duration of life	157 "
Probable error of average	±22 "
Variability of the duration of life	18 per cent
Median duration of life (50 per cent dead)	830 days
Probable error of median	±28 "

TABLE VII.

Mortality of Tethelin-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
400–500	1	6	6	496
500–600	1	6	12	596
600–700	6	38	50	659
700–800	0	0	50	—
800–900	3	19	69	859
900–1,000	1	6	75	984
1,000–1,100	4	25	100	1,051

Total No. of animals	16
Average duration of life	800 days
Standard deviation of duration of life	158 "
Probable error of average	±27 "
Variability of the duration of life	20 per cent
Median duration of life (50 per cent dead)	indeterminate

TABLE VIII.

Mortality of Lecithin-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
300–400	2	6.0	6.0	348
400–500	4	12.5	19.0	435
500–600	6	19.0	37.5	552
600–700	4	12.5	50.0	640
700–800	3	9.0	59.0	765
800–900	4	12.5	72.0	848
900–1,000	4	12.5	84.0	973
1,000–1,100	2	6.0	91.0	1,031
1,100–1,200	3	9.0	100.0	1,149

Total No. of animals	32
Average duration of life	731 days
Standard deviation of duration of life	243 "
Probable error of average	±29 "
Variability of the duration of life	33 per cent
Median duration of life (50 per cent dead)	640 days
Probable error of median	±36 "

TABLE IX.

Mortality of Lecithin-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days.				*days*
200–300	1	3	3	247
300–400	2	6	9	374
400–500	5	15	24	456
500–600	4	12	35	521
600–700	5	15	50	658
700–800	7	21	70	746
800–900	5	15	85	846
900–1,000	3	9	94	960
1,000–1,100	2	6	100	1,022

Total No. of animals	34
Average duration of life	677 days
Standard deviation of duration of life	202 "
Probable error of average	±23 "
Variability of the duration of life	30 per cent
Median duration of life (50 per cent dead)	658 days
Probable error of median	±29 "

TABLE X.

Mortality of Cholesterol-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
200–300	1	3	3	293
300–400	1	3	6	329
400–500	1	3	9	490
500–600	4	11	20	556
600–700	5	14	34	678
700–800	6	17	51	768
800–900	11	31	83	861
900–1,000	4	11	94	936
1,000–1,100	1	3	97	1,077
1,100–1,200	1	3	100	1,125

Total No. of animals	35
Average duration of life	764 days
Standard deviation of duration of life	178 "
Probable error of average	±20 "
Variability of the duration of life	23 per cent
Median duration of life (50 per cent dead)	765 days
Probable error of median	±25 "

TABLE XI.

Mortality of Cholesterol-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
days				*days*
200–300	2	6	6	263
300–400	2	6	12	357
400–500	4	13	25	449
500–600	4	13	38	555
600–700	9	28	66	652
700–800	2	6	72	754
800–900	4	13	85	842
900–1,000	1	3	87	925
1,000–1,100	4	13	100	1,030

Total No. of animals	32
Average duration of life	658 days
Standard deviation of duration of life	220 "
Probable error of average	±26 "
Variability of the duration of life	33 per cent
Median duration of life (50 per cent dead)	595 days
Probable error of median	±33 "

TABLE XII.

Proportion of Animals Surviving to Various Ages.

Class of animals.	Percentage surviving to Days.								
	300	400	500	600	700	800	900	1,000	1,100
Males.									
Normal	94	94	94	81.5	69	50	34	6	0
Pituitary	100	97	94	88	78	52	23	13	3
Cholesterol	97	94	91	80	66	49	17	6	3
Lecithin	100	94	81	62.5	50	41	28	16	9
Tethelin	100	100	100	91	78	78	48	26	4
Females.									
Normal	97	91	85	69	60	32	16	13	0
Pituitary	100	94	81	72	63	43	9	0	0
Cholesterol	94	88	75	62	34	28	15	13	0
Lecithin	97	91	76	65	50	30	15	6	0
Tethelin	100	100	94	88	50	50	31	25	0

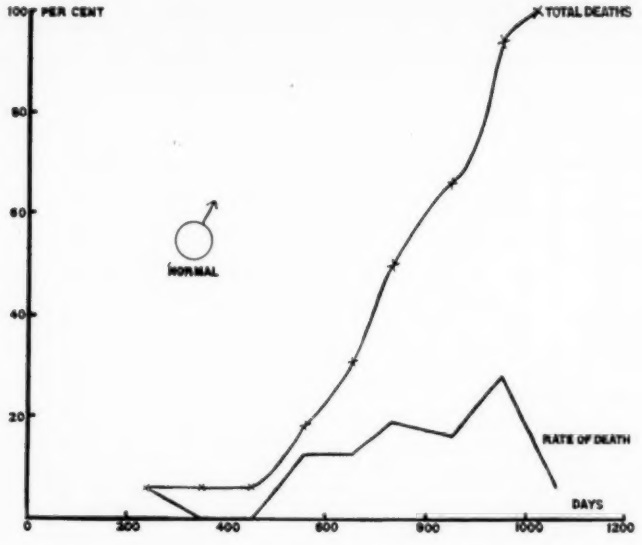

FIG. 1. Mortality curves for normal male white mice.

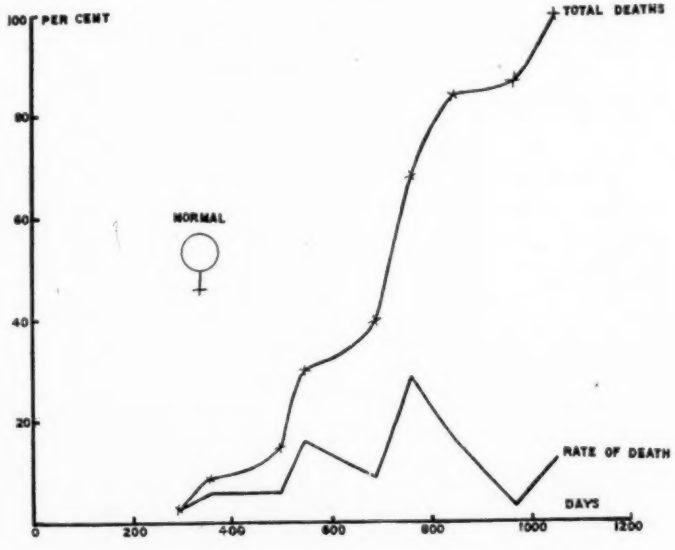

FIG. 2. Mortality curves for normal female white mice.

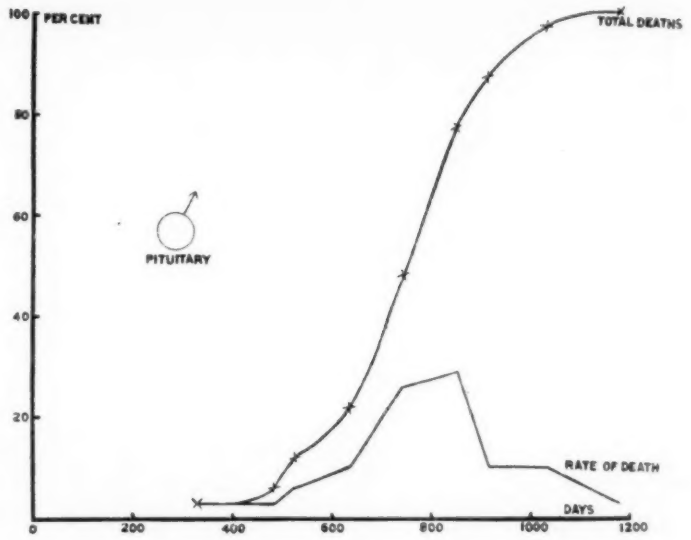

FIG. 3. Mortality curves for pituitary-fed male white mice.

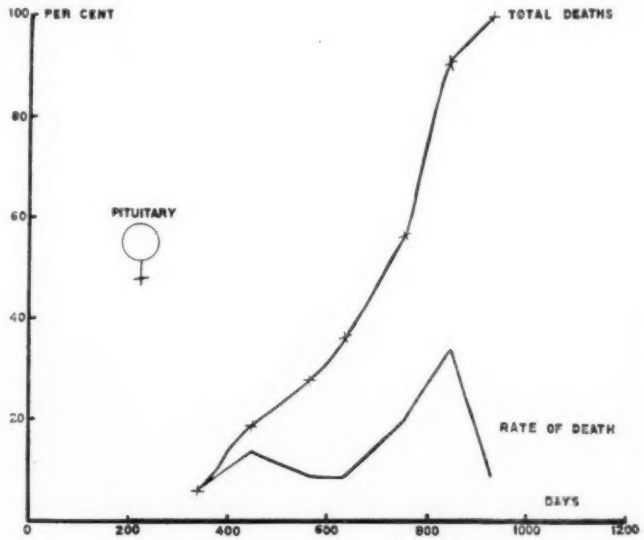

FIG. 4. Mortality curves for pituitary-fed female white mice.

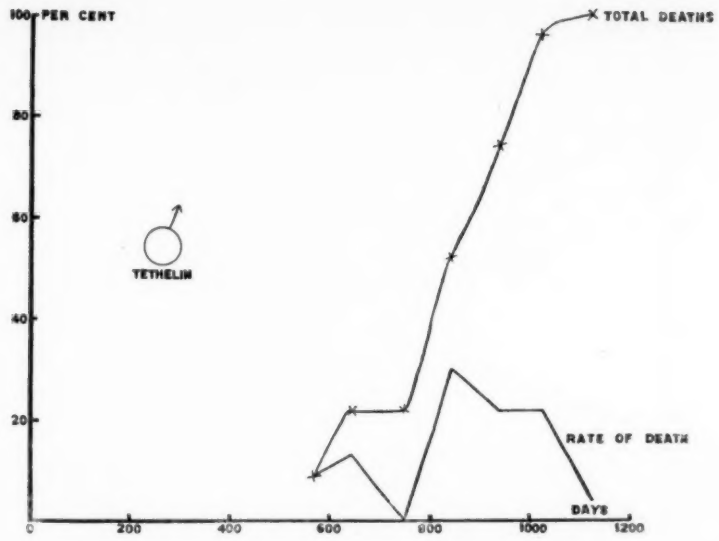

FIG. 5. Mortality curves for tethelin-fed male white mice.

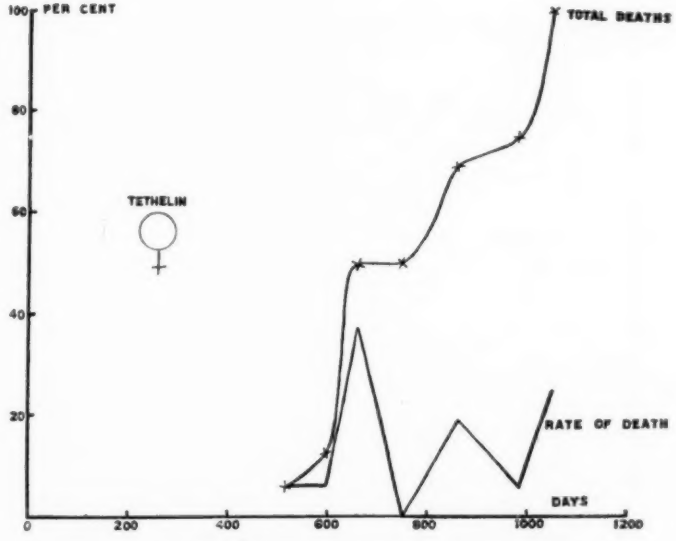

FIG. 6. Mortality curves for tethelin-fed female white mice.

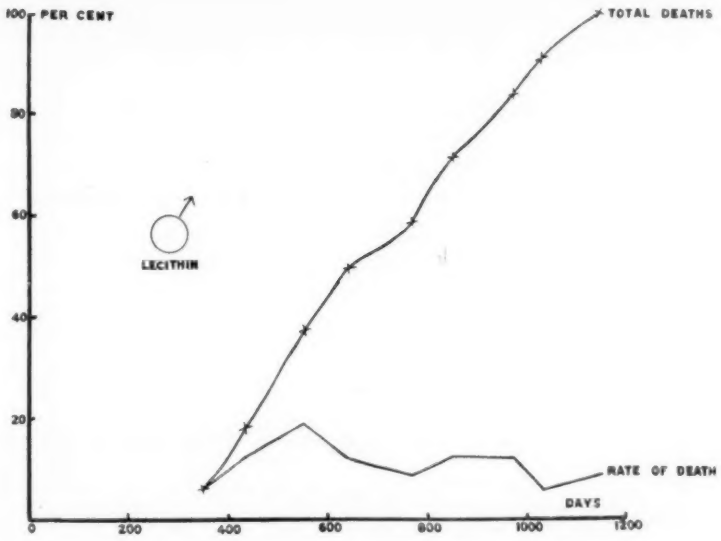

FIG. 7. Mortality curves for lecithin-fed male white mice.

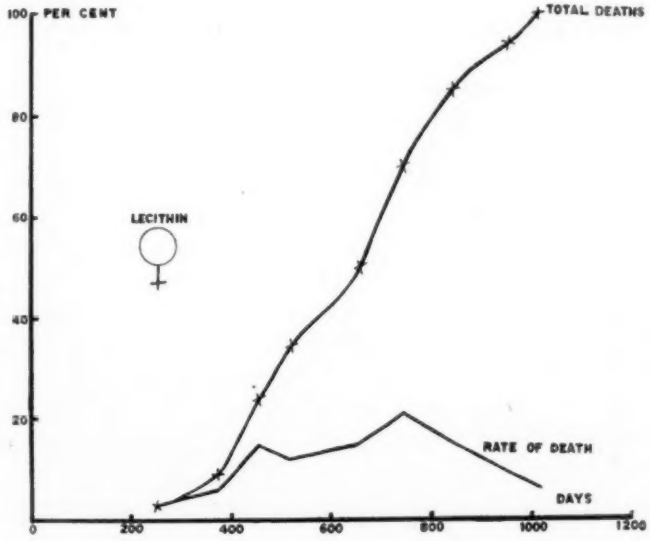

FIG. 8. Mortality curves for lecithin-fed female white mice.

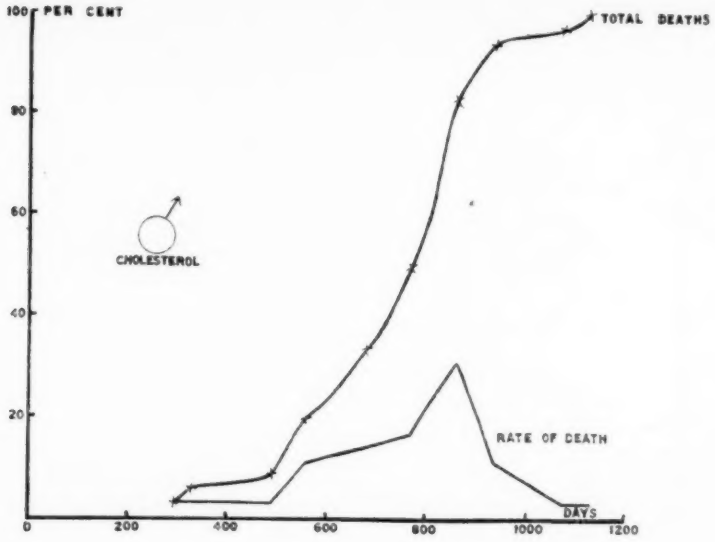

FIG. 9. Mortality curves for cholesterol-fed male white mice.

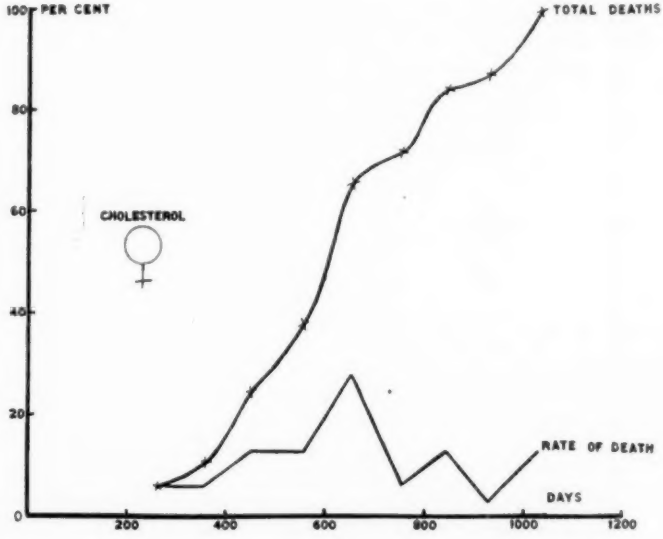

FIG. 10. Mortality curves for cholesterol-fed female white mice.

EXPERIMENTAL STUDIES ON GROWTH.

XIII. LESIONS EXHIBITED BY NORMAL, PITUITARY-, LECITHIN-, CHOLESTEROL-, AND TETHELIN-FED WHITE MICE AT THE OCCURRENCE OF NATURAL DEATH, WITH ESPECIAL REFERENCE TO THE INCIDENCE AND DEVELOPMENT OF SPONTANEOUS CANCER.

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Procedure.

The animals employed in the investigations described in the preceding articles of this series (Papers I to VI and X to XII) were invariably examined for lesions after the occurrence of natural death, except in a small proportion of cases in which delayed detection of death led to decomposition which rendered examination of the tissues useless or impossible.

Gross lesions or tumors manifest to the naked eye on examination of the organs were noted and sections of formaldehyde-hardened material were prepared and examined microscopically. No attempt was made to examine sections from a variety of organs and tissues as a routine procedure, because our facilities and the time available did not permit us to undertake such a task. For this reason a considerable proportion of incipient cases of nephritis, for example, as well as other lesions of interest and importance may have been overlooked. The carcasses of all the animals have been preserved in formaldehyde for further pathological studies. We feel confident that nearly all cases of carcinoma and other new growths were detected and identified except in the small proportion of cases in which postmortem examination of the tissues was impossible.

In a certain proportion of cases the naked eye examination of the tissues revealed no obvious lesions or cause of death. Such cases frequently occurred in very old, senescent animals, in which death was probably due to simultaneous atrophic degeneration of a number of organs.

The results of our postmortem examinations are summarized in Tables I and II and are given in detail in Tables III to XII. No deaths prior to 210 days are included in the tables, such deaths, as explained in the preceding article, being regarded as essentially accidental in origin.

Infections.

Local infections occurred in and were responsible for the death of a relatively large proportion of the males. This is attributable to injuries received in fighting, especially in the neighborhood of the penis and testicles. Late in life, and frequently after complete healing of the traumatisms as far as could be judged externally, these lesions became the center of localized infections, which frequently terminated life by blocking the passage of the urethra.

General infections (pneumonia and β-paratyphoid) were rare, since especial precautions were taken to prevent infection from one animal or cage to another. Among all the males an average of slightly over 4 per cent died, usually late in life, from generalized infections, and among the females less than 1 per cent.

Carcinoma.

Tumors accounted for the death of a great proportion of the animals of both sexes and of all dietary classes. Carcinomas were much more abundant in the females than in the males, occurring in 27 per cent of all the males and 39 per cent of all the females. This figure corresponds closely with the average incidence found by Lathrop and Loeb in a variety of different strains of mice.[1]

No definite effect of the dietary administrations upon the frequency of incidence of carcinoma was observed. This fact is significant when the markedly accelerative action of cholesterol

[1] Lathrop, A. E. C., and Loeb, L., *J. Cancer Research*, 1916, i, 1.

TABLE I.

Average Age at Death in Animals with the Following Lesions.

Class of animals.	Carcinoma.	Sarcoma.	Degenerative lesions.	Average duration of life.
		Males.		
	days	*days*	*days*	*days*
Normal	826	717	998	767
Pituitary	823	765	855	792
Lecithin	743	681	874	731
Cholesterol	848	586	837	764
Tethelin	958	766	1,065	866
		Females.		
Normal	721	746	—	719
Pituitary	689	743	—	704
Lecithin	674	725	634	677
Cholesterol	590	506	871	658
Tethelin	970	774	—	800

TABLE II.

Percentage of Animals Exhibiting Certain Lesions at Death.

Class of animals.	Local infection.	General infection.	Carcinoma.	Sarcoma.	Degenerative lesions.	No lesions ascertained.
			Males.			
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Normal	28	3	25	19	9	16
Pituitary	26	3	29	10	9	23
Lecithin	9	9	22	19	12	28
Cholesterol	3	3	26	3	29	37
Tethelin	4	4	35	26	4	26
			Females.			
Normal	3	0	37	47	0	13
Pituitary	3	3	51	20	0	23
Lecithin	3	0	32	26	18	21
Cholesterol	3	0	50	12.5	21	12.5
Tethelin	0	0	25	44	0	31

and tethelin upon the growth of inoculated carcinomas is recalled,[2] and it demonstrates decisively the truth of the generalization to which other investigators have been led,[3] that the conditions favorable to the *incidence* of carcinoma are quite distinct from those which are favorable to its *growth*.

No definite effects were observed upon the duration of life of the animals which exhibited carcinomatous neoplasms at death in any of the dietary classes except those which received tethelin (4 mg. daily). In these the prolongation of life of the animals exhibiting carcinomas at death was even more marked than the prolongation of life of the average tethelin-fed animals (132 days in the males, and 249 days, or 33 per cent of the whole normal duration of life, in the females). Since no other classes exhibited the slightest tendency to prolongation of life of the carcinomatous individuals, we must regard this effect as distinctively attributable to the administration of tethelin.

Since carcinoma is essentially a disease which accompanies a measure of senescence, we may infer that the delay in the death of the carcinomatous animals was due to the fact that the onset of senescence in the tethelin-fed animals is markedly deferred, and the spontaneous origin of carcinoma suffered even more than proportionate delay. The fact that the delay in the incidence of carcinoma was *more* than proportionate to the prolongation of the average life may be correlated with the fact that senescent loss of weight, when it does initiate in tethelin-fed animals, is less rapid than it is in normal animals (cf. Figs. 3 and 4 in Paper XI).

Lathrop and Loeb, in their investigations upon tumor incidence and tumor age in mice[1] do not report the death of any animals having tumors at ages exceeding 2 years, and our observations upon mice of all dietary classes, other than the tethelin-fed groups, support the conclusion that the oldest animals do not develop carcinoma. In the tethelin-fed groups, however, the reverse was true, and the carcinomatous growths were encountered most abundantly in the animals which survived the longest.

[2] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344; 1915, xxi, 280; 1916, xxiii, 631. Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1915, xxi, 309.

[3] Woglom, W. H., *J. Exp. Med.*, 1915, xxii, 766. Robertson, T. B., and Burnett, T. C., *J. Cancer Research*, 1918, iii, 75.

The result could not have been influenced by hereditary factors, such as the accidental selection of animals descended from a small number of common ancestors transmitting these peculiarities to their offspring, for the male and female groups of tethelin-fed animals were born in August and October, 1914, respectively, while the cholesterol-fed groups which were selected from the same stock and did not display this peculiarity were born in September and October, 1914; that is, subsequent to the birth of the tethelin-fed males and prior to the birth of the females.

Even more marked than the delay in the *incidence* of carcinoma, was the delay of its *growth* in the tethelin-fed animals. A majority of the animals exhibiting carcinoma in other classes died as a result of growth of the tumor, and consequent interference with the functions of the organs in its vicinity. In the tethelin-fed groups of animals, on the contrary, death in nearly all cases occurred from other and undefined causes, possibly diffuse senescent atrophy of one or more organs, not evident to the naked eye, and the carcinoma was confined to one or more minute spots of from 1 to 6 mm. diameter in the lungs.

This result was very unexpected, since in animals which are afflicted with *inoculated* carcinoma, the administration of tethelin causes marked acceleration of the growth. A reason for this discrepancy may possibly reside in the differing locality of primary incidence in tethelin-fed as compared with the other classes of animals. In all the other classes the primary growth generally occurred in the axilla or in the groin. In the tethelin-fed animals the primary growth did not, as a rule, occur in these localities, or at all events did not make itself grossly manifest there, the only growth, in the majority of cases, being found in the lungs.

Sarcoma.

Several types of sarcoma were encountered, but the most common was a lymphosarcoma, probably identical with the lymphoadenoma of mice described by Haaland[4] and by Murray.[5]

[4] Haaland, M., *Ann. Inst. Pasteur*, 1905, xix, 165.

[5] Murray, J. A., Third scientific report of the investigations of the Imperial Cancer Research Fund, London, 1908, p. 69.

It appears, however, to be more abundant in our stock than in the strains of mice examined by Murray, who reports only four cases in a large number of animals.

This tumor consisted of masses of lymphoid cells widely distributed in the body and sometimes accompanied by lymphoid infiltration of the organs, resembling Hodgkin's disease. In one instance masses of lymphoid tissue were found even in the brain. The distribution of this tumor differed widely from that of carcinoma, and diagnosis, although always confirmed by the microscope, could confidently be made with the naked eye. The lymphoid masses occurred chiefly in the mesenteric glands and the glands adjacent to the ureters and the kidneys, while the thymus was almost invariably the site of a larger or smaller growth and the great majority of animals displayed a marked splenomegaly. The carcinomas, on the contrary, occurred in the subepithelial regions in the groin and axilla and metastasized into the lungs, the thymus remaining unaffected.

The frequency of the incidence of sarcoma was decidedly reduced by the administration of cholesterol; it was one-sixth of the average in all other classes of males and one-third of the average in all other classes of females. On the other hand, the frequency of the incidence of sarcomas (almost all lymphosarcomas) was greater in the tethelin-fed animals, being twice the average of all other classes in both males and females. In the females, however, the incidence did not exceed that found in the normal females. Probably this slightly increased frequency of incidence of sarcomas in the tethelin-fed animals was due merely to the delay in the incidence of carcinoma, permitting a greater proportion of sarcomas to develop.

The duration of life of the small proportion of cholesterol-fed animals which developed sarcomas was decidedly reduced, 131 days below the average duration of life of the normals in the males and 240 days in the females. In other words, sarcoma developed early or not at all. The duration of life of the tethelin-fed animals which developed sarcomas was slightly above normal.

In all groups of male animals the duration of life of those which developed sarcoma was less than that of the animals which developed carcinoma. In each of the groups of female animals, with the exception of those receiving cholesterol or tethelin, the

reverse was the case. In all classes of male animals the average duration of life of the animals which developed sarcoma was less than the average duration of life for all the male animals in the class. In each of the classes of female animals, with the exception of the cholesterol- and tethelin-fed groups, the reverse was the case. There is therefore a correlation between sex and the relative and absolute ages of incidence of these two types of tumors, and the exception afforded by the cholesterol- and tethelin-fed groups may be correlated with the abnormal time relations of growth in these two groups, which resembled one another, but differed markedly from those displayed by the remaining classes of animals (cf. Paper XI).

In eight of the ten groups of animals studied, carcinomas were more numerous than sarcomas.

Degenerative Lesions.

Degenerative lesions, usually of the kidney, but occasionally of the liver or other organs, were frequent in the animals of advanced age, and more especially in the animals which received cholesterol. The unusual frequency of degenerative lesions in the cholesterol-fed animals must be attributed to the extensive deposits of cholesterol which form in various tissues of animals fed unusual quantities of cholesterol,[6] and which give rise to lesions which subsequently become centers of degenerative changes. The presence of such deposits in the livers and adrenals of our animals was confirmed by Dr. C. H. Bailey, whom we wish to thank for examining a number of specimens.[7]

Degenerative lesions in the kidneys (interstitial nephritis) were most abundant in the cholesterol-fed animals, the percent-

[6] Chalatow, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 85. Anitschkow, N., *ibid.*, 1913, lvi, 379; 1914, lvii, 201. Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 68; 1915–16, xiii, 60.

[7] It is of interest to note that Chalatow and Anitschkow failed to elicit the formation of deposits of cholesterol in the liver by administering cholesterol by mouth to rats, although they readily obtained them in rabbits. This may possibly indicate a difference in the lipoid metabolism of rats and mice, or more probably may merely be traceable to differences in the dosages administered.

age of observed cases being four times the average for all other classes in the cholesterol-fed males and six times the average for all other classes in the cholesterol-fed females. This fact is remarkable because, according to the authors cited, manifest deposits of cholesterol do not occur in the kidneys of normal animals receiving cholesterol by mouth.[8] It has, however, been pointed out by Weltmann and Biach[9] that when the kidneys have been injured, as, for example, in uranium nephritis, cholesterol is deposited in the injured cells and may doubtless cause exaggeration of the injury. We can understand, therefore, how administration of cholesterol might increase and make prominent lesions which would otherwise have remained latent or subacute until death.

Facilities did not permit a special investigation of the question of cholesterol deposits in the kidneys, and furthermore administration of cholesterol, through lack of a sufficient supply, was discontinued a year before the majority of the most marked cases of nephritis developed. By this time the original deposits of cholesterol might be expected to have disappeared, although the lesions caused by them would presumably have remained.

TABLE III.

Normal Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	720	28
" general	720	3
Carcinoma	826	25
Sarcoma	717	19
Degenerative lesions: kidneys	998	9
No cause ascertained	689	16
Average duration of life	767	

[8] Anitschkow, N., *Deutsch. med. Woch.*, 1913, xxxix, 741.

[9] Weltmann, O., and Biach, P., *Z. exp. Path. u. Therap.*, 1913, xiv, 367.

TABLE IV.

Normal Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	1,065	3
" general	—	0
Carcinoma	721	37
Sarcoma	746	47
Degenerative lesions	—	0
No cause ascertained	524	13
Average duration of life	719	

TABLE V.

Pituitary-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	515	26
" general	1,032	3
Carcinoma	823	29
Sarcoma	765	10
Degenerative lesions		
Kidneys	835	6
Liver	895	3
No cause ascertained	875	23
Average duration of life	792	

TABLE VI.

Pituitary-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	477	3
" general	898	3
Carcinoma	689	51
Sarcoma	743	20
Degenerative lesions	—	0
No cause ascertained	709	23
Average duration of life	704	

TABLE VII.

Lecithin-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	515	9
" general	564	9
Carcinoma	743	22
Sarcoma	681	19
Degenerative lesions		
Kidneys	1,031	9
Stomach	402	3
No cause ascertained	807	28
Average duration of life	731	

TABLE VIII.

Lecithin-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	729	3
" general	—	0
Carcinoma	674	32
Sarcoma	725	26
Degenerative lesions		
Kidneys	637	6
Ulcers	523	9
Intestine	961	3
No cause ascertained	650	21
Average duration of life	677	

TABLE IX.

Cholesterol-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	816	3
" general	329	3
Carcinoma	848	26
Sarcoma	586	3
Degenerative lesions		
Kidneys	811	26
Liver	1,077	3
No cause ascertained	692	37
Average duration of life	764	

TABLE X.

Cholesterol-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	925	3
" general	—	0
Carcinoma	590	50
Sarcoma	506	12.5
Degenerative lesions		
Kidneys	834	9
Ovary	846	9
Liver	1,057	3
No cause ascertained	641	12.5
Average duration of life	658	

TABLE XI.

Tethelin-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	815	4
" general	544	4
Carcinoma	958	35
Sarcoma	766	26
Degenerative lesions, kidneys	1,065	4
No cause ascertained	871	26
Average duration of life	866	

TABLE XII.

Tethelin-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	days	*per cent*
Infections, local	—	0
" general	—	0
Carcinoma	970	25
Sarcoma	774	44
Degenerative lesions	—	0
No cause ascertained	703	31
Average duration of life	800	

EXPERIMENTAL STUDIES ON GROWTH.

XIV. FURTHER EXPERIMENTS ON THE INFLUENCE OF TETHELIN UPON THE GROWTH OF THE WHITE MOUSE.

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(Received for publication, January 3, 1919.)

In 1914 two experiments on the influence of tethelin upon the growth of the white mouse were begun. In one of them, consisting of twenty-four animals, the administration (4 mg. per day) was continuous, while in the other, consisting of sixteen animals, the administrations were discontinuous, being confined to three periods of 4 weeks each in the first 30 weeks of life. The results of these experiments, up to the 60th week of life, were reported in 1916 in Paper IV of this series,[1] while the results from the initiation of the administrations until the natural death of the animals are reported in Paper XI.

Further experiments were not conducted at that time, owing to lack of facilities for the production of sufficient tethelin for daily administration to so many animals. In 1916 more abundant supplies of tethelin became available and a third and fourth group of animals were started upon tethelin, and in 1917 a fifth, comprising a total of 134 animals in five different groups: three of females (total, 88 animals) and two of males (total, 46 animals).

The effectiveness of discontinuous administrations of tethelin in producing the characteristic deformations of the growth curve that accompany the administration of tethelin, led to the investigation of the effects of a single relatively brief period of administration in two of the three new experimental groups; namely, in a group of 24 females started in 1916, and a group of 22 males started in 1917. In these experiments tethelin (4 mg. per day) was administered for 8 weeks only, from the 4th to the 12th

[1] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 397.

week of age, and then discontinued for the remainder of the animal's life. In the remaining experiment 48 females received tethelin continuously (4 mg. per day).

The results are shown in Tables I to III and Figs. 1 to 3. The figures show in every case the characteristic deformation of the growth curve, resulting in a marked concavity in what would normally be the most rapidly rising part of the curve. In no other experimental group except those receiving cholesterol, and in none of the six groups of normals studied during this period (cf. Paper X) has this marked concavity in the curve of growth been noted, and it has occurred in five separate groups of tethelin-fed animals.

The concavity in the curve of growth is the expression of preliminary retardation preceding sexual maturity, followed by a secondary or compensatory acceleration accompanying and succeeding sexual maturity.

In our previous reports we inclined to the view that the acceleration, in common with the retardation, was directly due to tethelin. We now believe that while the retardation is due to tethelin, the acceleration is due to compensatory factors which develop in the animal itself in response to the abnormal dosage of the active principle of the anterior lobe of the pituitary body. We prefer this view since the compensatory acceleration is more intense when the retarding factor, tethelin, is discontinued after the 12th week of age and 8th week of the administration. This fact is evident from Figs. 2 and 3.

In adult animals which receive tethelin continuously, the compensatory acceleration disappears, and the curves of the tethelin-fed and normal animals become nearly parallel, the normals increasing in weight, in fact, more rapidly than the tethelin-fed animals until the onset of senescence. But in animals which have only received tethelin from the 4th to the 12th week the acceleration continues in most marked degree. The effects of brief administration upon late growth will be reported subsequently but it may be stated that by this means we have succeeded in producing a number of exceptionally large animals.

In the females of 1916, receiving the brief administration of tethelin, the secondary acceleration led very early (at 11 weeks) to intersection of the normal curve by the curve of the tethelin-

fed animals, retardation being displayed only by the acceleration which succeeded it. In the males of 1917 the intersection with the curve of the 1916 normals[2] occurred at a later period (18 weeks). The growth curves of animals receiving tethelin continuously do not intersect the normal curve until advanced old age, when the delay of senescence in the tethelin-fed animals confers an advantage upon them relatively to the normals.

Since the direct action of tethelin would thus appear to consist exclusively, so far as the whole animal is concerned, of retardation of growth, the question presents itself how the acceleration of growth of epithelial tissues *in situ* arises which is noted in the effects of tethelin upon the healing of superficial wounds[3] and upon the growth of inoculated carcinoma.[4] Two possibilities suggest themselves; namely, that tethelin accelerates the growth of epithelial tissues and directly or indirectly retards the growth of the other and collectively more bulky tissues, or that tethelin retards the growth of all tissues, but indirectly accelerates the growth of epithelial tissues *in situ* by disproportionately retarding the growth of other tissues, and thus favoring the epithelial tissues in the competition for nutritive materials. Between these two alternatives a decision may be reached only by experiments *in vitro*, for which the opportunity has hitherto been lacking.

[2] No 1917 normals were available for comparison, but since in 1916 our stock was already approaching a stable type (cf. Paper X) it is probable that the 1917 normals would not have differed appreciably from those of 1916.

[3] Robertson, T. B., *J. Am. Med. Assn.*, 1916, lxvi, 1009. Barney, E. L., *J. Lab. and Clin. Med.*, 1917–18, iii, 480.

[4] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1916, xxiii, 631.

TABLE I.

Tethelin-Fed Females, 1916. Continuous Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	30	10.23	−2.20	5.8	19.6
5	48	11.78	−1.89	5.4	22.0
6	48	13.26	−1.82	4.8	19.4
7	48	14.65	−1.33	3.8	18.4
8	48	15.85	−1.09	3.2	17.3
9	48	16.59	−0.91	2.8	14.5
10	48	17.03	−0.70	2.4	13.5
11	48	17.36	−0.83	2.4	14.2
12	48	17.70	−0.72	2.1	13.8
13	48	18.07	−0.49	1.4	14.1
14	48	18.31	−0.92	2.4	14.6
15	48	18.67	−0.75	2.0	15.6
16	48	18.91	−0.99	2.7	14.9
17	48	19.22	−0.97	2.5	15.1
18	48	19.21	−0.85	2.1	15.4
19	48	19.35	−0.96	2.4	15.3
20	48	19.66	−1.22	3.0	15.4
21	48	19.93	−0.92	2.2	15.2
22	48	19.86	−1.03	2.5	14.8
23	48	20.13	−1.09	2.5	14.8
24	48	20.16	−1.23	2.9	14.9
25	48	20.39	−1.20	2.5	15.1
26	48	20.54	−1.22	2.5	13.9
27	48	20.56	−1.44	3.0	13.7
28	48	20.58	−2.13	4.4	14.0
29	46	20.44	−2.33	4.5	14.8
30	45	20.76	−2.07	4.2	14.7
32	48	21.38	−1.73	3.5	14.0
34	48	21.81	−1.36	2.8	14.4
36	48	22.10	−1.55	3.1	15.3
38	48	22.31	−1.59	2.9	15.3
40	48	22.67	−1.26	2.2	16.6
42	47	23.09	−1.39	2.4	14.3
44	47	23.78	−0.22	0.4	13.8
46	47	23.55	−0.69	1.4	14.3
48	47	24.04	−0.03	0.1	15.3
50	47	24.20	+0.16	0.3	15.2

TABLE II.

Tethelin-Fed Females, 1916. Brief Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	13	10.54	−1.89	5.6	9.0
5	24	12.63	−1.04	2.4	20.0
6	24	13.92	−1.16	2.7	16.2
7	24	15.54	−0.44	1.0	15.9
8	24	16.46	−0.48	1.2	15.0
9	24	17.23	−0.27	0.6	14.8
10	24	17.58	−0.15	0.4	14.2
11	24	18.35	+0.16	0.4	13.4
12	23	19.46	+1.04	2.4	13.6
13	23	19.91	+1.35	3.4	11.8
14	23	20.43	+1.20	2.7	13.3
15	23	20.63	+1.21	2.7	12.6
16	23	21.04	+1.14	2.6	11.7
17	23	21.46	+1.27	2.8	11.4
18	23	21.98	+1.92	4.3	12.0
19	23	22.48	+2.17	4.6	12.1
20	23	22.20	+1.32	2.9	11.9
21	23	22.70	+1.85	3.7	12.7
22	23	22.67	+1.78	3.6	12.4
23	23	22.80	+1.58	3.2	12.1
24	23	23.22	+1.83	3.7	12.3
25	23	23.35	+1.76	3.1	13.0
26	23	23.65	+1.99	3.4	12.6
27	23	23.52	+1.52	2.5	14.3
28	23	23.59	+0.88	1.4	15.1
29	23	23.52	+0.75	1.2	13.3
30	23	24.00	+1.17	1.9	14.7

TABLE III.

Tethelin-Fed Males, 1917. Brief Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
wks.		*gm.*			*per cent*
4	14	10.50	−1.79	3.4	18.8
5	22	12.05	−1.20	2.3	21.0
6	22	13.82	−1.22	2.4	19.6
7	22	15.16	−1.67	3.4	17.0
8	22	15.57	−2.51	5.2	15.9
9	22	16.95	−2.05	4.3	14.3
10	22	17.86	−1.77	3.9	13.0
11	22	18.57	−1.39	3.2	11.0
12	22	19.75	−0.69	1.5	10.9
13	22	19.66	−1.26	2.8	9.8
14	22	20.84	−0.47	1.2	7.4
15	22	21.41	−0.42	1.0	8.3
16	22	21.84	−0.31	0.7	7.7
17	22	22.36	−0.06	0.1	6.8
18	20	23.18	+0.60	1.4	6.8
19	20	23.58	+0.87	2.1	7.1
20	20	24.08	+1.06	2.5	7.3
21	20	24.45	+1.35	3.2	7.5
22	20	24.68	+1.26	2.9	7.7
23	20	25.05	+1.56	3.5	7.9
24	20	25.43	+1.80	4.5	7.1
25	20	25.80	+1.95	4.6	8.1
26	20	25.80	+1.70	4.3	6.5
27	20	25.90	+1.59	3.9	7.6
28	20	25.95	+1.64	3.7	7.9
29	19	25.97	+1.32	3.3	6.9
30	20	26.65	+1.74	3.7	7.8

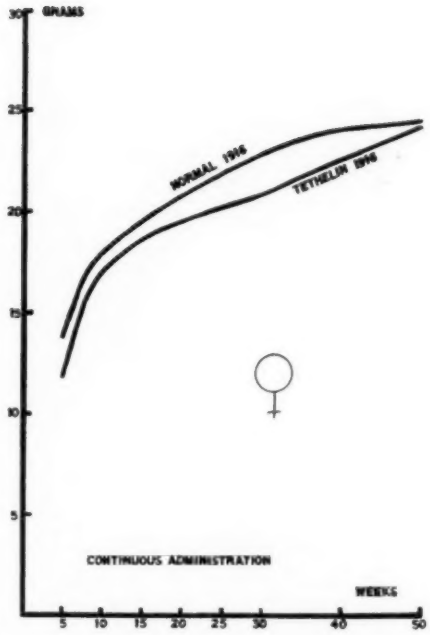

FIG. 1. Influence of tethelin upon the growth of female white mice. Administration continuous. Dosage 4 mg. per day.

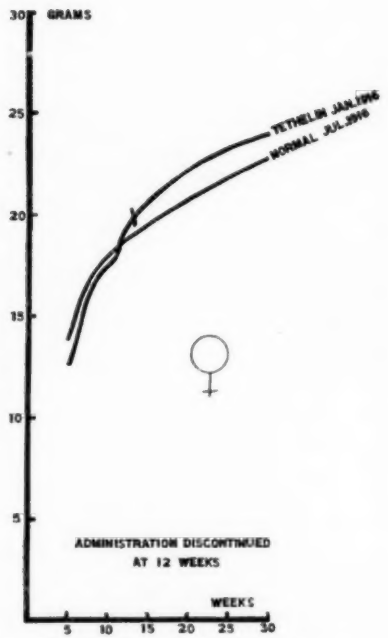

FIG. 2. Influence of tethelin upon the growth of female white mice. Administration discontinued at 12 weeks (indicated by vertical cross mark). Dosage 4 mg per day.

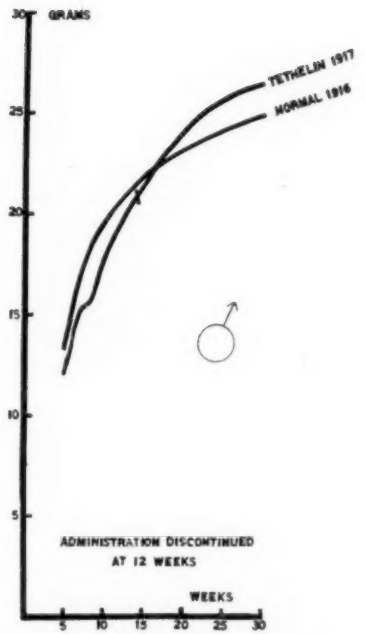

FIG. 3. Influence of tethelin upon the growth of male white mice. Administration discontinued at 12 weeks (indicated by vertical cross mark.) Dosage 4 mg. per day.

ABSORPTION SPECTRA OF ACID HEMATIN, OXYHEMOGLOBIN, AND CARBON MONOXIDE HEMOGLOBIN. A NEW HEMOGLOBINOMETER.

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(Received for publication, January 24, 1919.)

This paper contains spectrophotometric data on two hemoglobin derivatives. The transmissions of solutions of oxyhemoglobin, acid hematin, and carbon monoxide hemoglobin for light in the visible and ultra-violet portions of the spectrum are given. The data have a certain abstract interest. They serve to correlate properly spectrophotometric values with oxygen capacity. The immediate purpose in securing them was that they might serve as a basis for work on colorimetric hemometers, a problem which is discussed at the end of this paper. The optical properties of a new hemoglobinometer are described.

HISTORICAL.

The visible bands of oxyhemoglobin first described by Hoppe-Seyler (1) have received considerable attention, more particularly as regards their position. Soret (2) was the first to recognize the great ultra-violet band of oxyhemoglobin, a band which he observed with a fluorescing eyepiece. Later Gamgee (3) using a quartz spectrograph and the sun as a source, made a more extensive investigation of the ultra-violet absorption spectra. He showed that the great ultra-violet band was common to oxyhemoglobin, reduced hemoglobin, CO- and NO-hemoglobin, hemochromogen, hematin hydrochloride, methemoglobin, and hematoporphyrin. Gamgee did not find such a band in the spectra of bilirubin, hydrobilirubin, or urobilin. A paper by Rost, Franz, and Heise (4) contains excellent plates of the spectra of many of the hemoglobin derivatives, together with an extensive summary of earlier work. Lewin, Miethe, and Stenger (5) have also published extensive qualitative spectrographic data of this sort.

Rollett (6) gives a picture, only roughly correct, showing the change in width of the absorption bands of oxyhemoglobin with dilution. Vier-

ordt (7) was the first to study the quantitative absorption of these compounds throughout the visible spectrum. His tables and curves were made on a very early type of spectrophotometer having large instrumental errors. Cherbuliez (8), in 1890, published certain spectrophotometric data on the hemoglobins which he had made with an improved instrument. His absorption curves given for the more central portions of the visible spectrum are more nearly correct. The α-band of oxyhemoglobin is given as shallower than the β-band, an error difficult to avoid because of the disturbing effect of the very high transmission to the red side of the α-band.

Aside from these two determinations of absorption curves there have been many determinations of certain absorption constants and relations, in part undertaken with the view of fixing a spectrophotometric method for the quantitative determination of oxyhemoglobin and other hemoglobins.

If T is the fraction of light of a given wave-length transmitted by a substance, then the equation $T = 10^{-\epsilon}$ defines a number ϵ known as an extinction coefficient. The extinction coefficent is the cologarithm of the transmission. It was first shown by Beer (9) that for a solution a simple absorption relation exists between the concentration, c, the thickness, t, and the extinction coefficient, ϵ; namely, $\frac{ct}{\epsilon} = \text{constant}$. This equation holds under a wide range of conditions. For oxyhemoglobin, carbon monoxide hemoglobin, and acid hematin it would seem at least to hold within the range of dilution here used (whole blood 1:100 to 1:2,000). Butterfield (10) has shown that the relation holds in the case of oxyhemoglobin for a range of dilution of whole blood 1:1 to 1:160. Reid (11) has shown that oxyhemoglobin forms a true solution.

While ϵ is understood to be the extinction coefficient for a single wave-length, practically it is the mean ϵ for a sufficiently small spectral interval, the interval given by the exit slit of the spectrophotometer. The value of the constant of the equation being determined at such an interval for a known concentration, c, this value furnishes a basis for the calculation of any c by the instrumental determination of the ϵ. By properly choosing the spectral interval and using concentrations giving extinction coefficients within a certain optimal range, the determination may be made to within 1 per cent. With uniformity in slit widths such determinations are absolute, variations between different in-

struments being only within observational errors of the individual instrument.

With the thickness of solution equal to 1 cm. it has become customary to designate the constants by the letter A. The ratio of two such constants determined for two separate spectral intervals is a ratio independent of the concentration and a characteristic of the substance.

$$\frac{A'}{A} = \frac{c'/c'}{c/c} = \frac{c}{c'}$$

The principal determination of the values of these constants A_0 and A_0' for oxyhemoglobin, are as follows:

Hüfner (12, 13)

$A_0 = 2.070$ (5540 to 5650 Ångström units)[1]

$A_0' = 1.312$ (5315 " 5425 " ")

$\frac{A_0}{A_0'} = 1.578.$

[1] The Ångström unit is one ten-millionth of a mm. or one ten-thousandth μ. The Fraunhofer lines often referred to as reference lines have the wave-lengths in Ångström units given below. The values due to Rowland are corrected on the basis of the accepted values for the primary standard cadmium line and the secondary standard iron arc lines of Fabry-Buisson (see Fowle, F. E., *Smithsonian Miscellaneous Coll., Publication 2269*, 1918, lxiii, No. 6):

	Å. u.		Å. u.		Å. u.		Å. u.		Å. u.		Å. u.
B	6867.2	E_2	5269.55	F	4861.35	L	3820.44	Q	3286.76	T	3020.65
C	6562.8	b_1	5183.62	G	4307.92 4307.75	M	3727.64	R	3181.26 3179.33	t	2994.42
D_1	5895.95	b_2	5172.69	h	4101.85	N	3581.19	S_1 S_2	3100.67 3100.31 3099.93	U	2947.89
D_2	5889.98	b_3	5169.05 5168.90	H	3968.48	O	3441.00 3440.61				
E_1	5270.39 5270.27	b_4	5167.51 5167.33	K	3933.68	P	3361.18	s	3047.61		

Cherbuliez (8)

$A_0 = 2.038$ (5530 to 5580 Ångström units)
$A_0 = 1.326$ (5360 " 5410 " ")
$\frac{A_0}{A_0'} = 1.537$

De Saint Martin (14) (polarization spectrophotometer)

$A_0 = 2.153$ (5572 to 5683 Ångström units)
$A_0' = 1.330$ (5380 " 5490 " ")
$\frac{A_0}{A_0'} = 1.621$

Bardachzi (15) (polarization spectrophotometer)

$A_0 = 2.094$ (5540 to 5650 Ångström units)
$A_0' = 1.334$ (5315 " 5425 " ")
$\frac{A_0}{A_0'} = 1.56$

Butterfield (16) (polarization spectrophotometer)

$A_0 = 1.87$ (5561 to 5646 Ångström units)
$A_0' = 1.18$ (5335 " 5420 " ")
$\frac{A_0}{A_0'} = 1.58$

Butterfield (17)

$\alpha_1 = 18.42$ (green mercury line, 5459 Ångström units)
$\alpha_2 = 21.00$ (yellow " lines, 5770 and 5790 Ångström units)
(α represents the Napierian cologarithm of the transmission of a 1 per cent solution.)

Butterfield's figures for A differ from those of other reliable observers by approximately 11 per cent. In determining the transmissions by oxyhemoglobin of the green mercury line or the two very adjacent and equal yellow mercury lines many of the technical difficulties connected with accurate spectrophotometric measurements disappear. Care no longer needs to be exercised in making wave-length measurements; there is much more latitude in the choice of slit width relations and various reflection errors are removed.

The α_1 and α_2 given by Butterfield might be transformed to correspond to the notation otherwise used:

$A_0^{546} = 1.25$
$A_0^{577,\ 579} = 1.096$

EXPERIMENTAL.

Apparatus.

The apparatus used was a Hilger sector photometer and quartz spectrograph, size C, giving a spectrum 18 cm. long, extending from 2100 to 7000 Ångström units. The light from a single source passed by means of appropriate quartz prisms along two separate and symmetrical paths, one above the other, in each of which was placed a so called rotating sector consisting of a circular disk with two symmetrical open sectors. The openings of the lower rotating sector were fixed in size and each was a quadrant. The openings in the other could be varied from 90–0°. To the sector photometer was bolted the absorption cell so that it could be easily demounted for filling and returned to exact alignment. The double absorption cell was made from a single piece of plate glass by drilling it with two holes slightly larger than the two beams of light and exactly centered on them. Through the edge of the glass plate entrance holes were drilled into the first ones. Windows of plane clear quartz were then fastened on each face of the glass plate with DeKhotinsky cement, thus making two cells each 1 cm. thick.

This construction gave a rigid alignment of the cells in symmetrical position with respect to the light paths. In the upper cell was placed distilled water and in the lower the absorption fluid. The absorption of the cell itself was thus corrected for at first hand. Opposite the lower cell was the fixed opening sector, and opposite the upper cell the variable sector.

The light coming along the two paths fell in juxtaposition on the collimator slit of the spectrograph and passed parallel in two beams to be dispersed into two spectra, one directly above the other, and so photographed on the plate. By varying the opening of the one sector the exposure of this comparison beam was cut down one step at a time. Twenty-four such simultaneous pairs of spectra could be taken on one plate. The wave-length at which a pair of spectra matched in density was the wave-length having an extinction coefficient corresponding to that sector opening. In a paper by Howe (18), and in one by myself (19), it has been quite conclusively shown that the extinction co-

efficient is the logarithm of the ratio of the fixed to the variable opening.

The plates used were the Wratten and Wainwright panchromatic of London, and of Eastman manufacture. They were given tank development according to the formulas accompanying them. The iron spark, or for the mid-visible spectrum sometimes the D lines of the sodium flame were always photographed on the plate as a comparison scale and used in connection with a wave-length scale furnished for the instrument by Hilger. The scale as checked against the spectra of several metals had errors less than the width of the smallest interval in which the two spectra could be compared.

The light source for the ultra-violet determinations was a condensed iron spark, and for the visible, a Nernst lamp with the filament vertical. At the beginning and end of each plate exposures were made with both cells filled with water in order to show that the two dispersed beams were of equal intensity, or to determine the relative sector openings required to give such equality. After the instrument was in adjustment a balance, with equal sector openings, could be obtained correct to within an extinction coefficient of $\frac{1}{100}$. To compensate for this error the sector settings required for balanced beams were included on each plate and the extinction coefficients corrected by the addition of this balance constant in each case. The plates often balanced to within a fraction of $\frac{1}{100}$. For the ultra-violet the balance was not determinable with as much precision, the smallest observable correction being about $\frac{1}{100}$. In making exposures it was possible for one sector to present its opening once oftener than the other sector. The sectors made 120 revolutions per minute. By increasing the exposures to 1 minute or more, sometimes to 10 minutes, and through repeated observations, this source of error was largely eliminated. A slit opening of 0.015 mm. was uniformly used. This corresponds with an interval of 3.5 Ångström units on the plate at 5500 Ångström units and of 0.3 Ångström units in the far ultra-violet.

The plates were read in a dimly lighted room by holding them above an illuminated white background and comparing the spectral pairs through a slit in a card, this slit being from 20 to 40 Ångström units wide (referred to 5500 region). In the visible region final readings were usually made through the smaller slit.

In the ultra-violet a larger slit was used sometimes which measured in width from 5 to 20 Ångström units. The wave-length position was read off at the center of the slit from the wave-length scale held in position on the plate at the spectrum in question.

Solutions.

The oxyhemoglobin, carbon monoxide hemoglobin, and acid hematin solutions were prepared by the dilution of human blood, usually my own. The diluting fluids were 0.1 per cent sodium carbonate, freshly prepared, 0.4 per cent ammonia water, and 0.1 N hydrochloric acid. For the preparation of carbon monoxide hemoglobin, the solution in the ammonia flask was immediately saturated with illuminating gas. The blood was drawn with a recalibrated Record syringe from a vein, and equal volumes were added to each of the fluids in the proportion 1 : 100. The estimation of hemoglobin content of the blood was then made on the material in the hematin flask and further dilution to about 1:200 made accordingly. The estimation of hemoglobin content was made on a colorimeter (20) using an artificial color match for hematin. The error in thus preparing the various solutions used from time to time so as to be of the same relative concentration was not large. It was eventually compensated for. In the case of the visible portion of the oxyhemoglobin absorption this was in part done by reducing each set of data to a uniform level through correcting it to correspond to a certain fixed extinction coefficient at the bottom of the α-band. The data for the visible portion of the acid hematin absorption were all obtained in a short interval of time and the errors as between the different solutions were small.

After the various portions of the absorption curves were determined they were corrected for relative concentrations and checked at various points by using solutions made up from blood of known oxygen capacity. The oxygen capacity of fresh oxalated human blood was determined by the method of Van Slyke (21) and simultaneously a 1 : 200 dilution made with the various diluting fluids, all glassware being recalibrated. The carbon monoxide absorption curve was wholly determined from two such solutions. It would have been easier and better to have had such material

for the determination of the other two curves. It was believed that in a complete redetermination an almost prohibitive amount of labor would have to be expended before changes in the curves as already computed would be justified. Certain corrections were however made. The question of absolute concentration of the solutions in gm. per liter will be considered later.

Oxyhemoglobin, when oxygen saturation has been reached, and carbon monoxide hemoglobin, when kept saturated with the gas, form under these circumstances true solutions, which, when kept cold, remain stable for at least a week, and probably for a month or more.

The acid hematin as thus prepared acts for the present purpose as if it formed a true solution. After several months particles settle out which thereafter cannot be brought again into stable suspension. This process is accelerated if the material is kept at room temperature and is most apparent in very dilute solutions. In case the hematin mixture is somewhat more concentrated than $\frac{1}{5}$% blood dilution, some particles settle out almost immediately. Under the above conditions of preparation the hematin solution assumes in an hour a spectrophotometric condition which remains nearly constant for several days and even for several weeks.

Relation of Time to Color Depth of Acid Hematin.

It is known (22) that the hematin solution does not assume its full depth of color immediately. In order to determine just how rapidly it does develop its full color depth we made readings on ten preparations of acid hematin from the bloods of as many individuals. The first set of readings was made during the 3rd minute after the preparation of the hematin, the second set during the 15th minute, another during the 30th minute, and a last set on the following day. As a result of a long previous experience I was able to assume that the solution had reached a stable condition within 24 hours. The most careful spectrophotometric determinations have not shown any demonstrable increase in color depth within the next 10 days. All readings were therefore calculated as percentages of these final values and averaged. It seemed reasonable to assume that the rate of formation of acid

hematin should be a rectangular hyperbola of the general form $xy = -c$, when x measures the time in minutes and y is the per cent by which the acid hematin falls short of its full color depth. Using the value for y corresponding to 30 minutes for x, the constant c of the equation is determined as 40. The values of y for 3 minutes and 15 minutes fit the curve given by the value of c very closely, justifying the assumption that the curve has the form of a hyperbola. Hence the equation for the color density of a newly prepared acid hematin solution is $xy = -40$, where x is the time in minutes and y the percentage short of full color. A graph (Curve D) of this equation is included among the curves of Fig. 2, the squares of the figure being ten units each.

The further trial was made of following a specimen through from 1 to 40 minutes, making readings continuously and averaging them by tens. The points so obtained lay very closely on the curve.

It will be seen that in 20 minutes $y = -2$ and in 40 minutes $y = -1$. In other words, the specimen then has reached 98 and 99 per cent respectively of its full density. It will also be seen that if the specimen is read in 2 minutes the density falls short 20 per cent, or is 80 per cent of the final figure.

The equation not only shows how rapidly the solution reaches its full density, but what correction should be added to a reading when it is made early.

Observations.

The ultra-violet portion of the acid hematin curve was determined from eight plates made on the bloods of four individuals. The sectors were set for variations of tenths in extinction coefficients. The various plates differed only slightly from each other. The data given are a mean of those derived from five plates of my own blood.

For the visible portion of the acid hematin curve seven plates were made using my own blood and with such settings that the extinction coefficient varied by hundredths. Three of the plates were made on the day the solutions were prepared, the others on the 2 following days. The plates were all consistent. They were read, their points plotted, and a mean curve was drawn

through the locus. The discrepancies ran not over 2 per cent on either side of this curve. The plates were again read to eliminate errors of judgment and the mean of the new locus was taken as the true curve, the discrepancies with two or three exceptions having become less than 1 per cent.

Determinations of the ultra-violet absorption of oxyhemoglobin were made on the same bloods as those used for acid hematin, ten plates being made. They showed variations in the region 3200 to 4300 Ångström units, which were not then understood. Some of the solutions showed transmissions in the region 3200 to 3800 Ångström units, which corresponded to extinction coefficients as much as 15 per cent greater than those given. The great ultra-violet band fell short of its full depth by similar amounts. It would seem that these variations were due to incomplete oxygenation of the hemoglobin.

Oxygenation resulting in the dilution of venous blood would require at the most 1 cc. of oxygen per liter of solution. At room temperature, water contains about 6 cc. of dissolved oxygen, and the oxyhemoglobin solution made therefrom would soon come into equilibrium with the atmosphere. In recently distilled water, equilibrium would be less readily established. It is probable that in the discrepancies observed solutions using freshly distilled water were not sufficiently shaken. The effect of blood plasma present in the proportion of about 1: 200 will be discussed later.

The ultra-violet portion of the oxyhemoglobin curve as obtained from certain apparently satisfactory plates was further checked on plates made from thoroughly oxygenated blood of known oxygen capacity. The form and depth of the curve at the great ultra-violet band centering on 4140 Ångström units was determined on solutions $\frac{1}{4}$ and $\frac{1}{10}$ as dense as those otherwise used. Here the curve was likewise carefully checked and discrepancies were small when the oxygen saturation was to be considered as satisfactory.

The visible portion of the oxyhemoglobin curve was at first determined from fourteen plates with my own blood. Except for short portions of the steep slopes of the curve the settings for extinction coefficients varied by hundredths. The plates having been correlated on the basis of the readings for the ex-

tinction coefficients at the maxima and minima points of the curve, all the plates were then read and their points plotted, the whole was rechecked for errors in judgment, and a curve was drawn through the locus. The discrepancies were somewhat less than those obtained in the visible portion of the acid hematin curve. The small extinction coefficients for the high transmission in the red were determined from the data of six plates, and the results checked by making readings with double the concentration.

The critical points of the curves for oxyhemoglobin and acid hematin, both in the ultra-violet and in the visible, were now checked on solutions of known oxygen capacity. About twenty plates in all were made for this purpose. They served at the same time to give data for the determination of the absolute concentration of the solutions to which the curves corresponded.

Incomplete data for the visible portion of the oxyhemoglobin curve had previously been obtained on a spectrophotometer at the physical laboratory of the United Gas Improvement Company. This was a Hilger quartz monochromatic illuminator coupled with a potassium photoelectric cell and galvanometer. A set of readings obtained with this apparatus for the region 4300 to 6000 Ångström units is consistent with that obtained by the photographic method except that the transmissions at the α- and β-bands, particularly the former, are not so low as they should have been. The dispersion of the instrument was too small for the purpose, and the slit width of 0.075 mm. used was too large. The blue and violet portion of the curve is, however, very closely checked and, as could be expected, the height of the α-β-peak was correctly observed.

I had originally made a determination of the absorption curves of oxyhemoglobin and acid hematin on a visual polarization spectrophotometer at the physical laboratory of the Corning Glass Works. These data were incorrect, the discrepancy being accounted for on the supposition of an error in the zero adjustment of the instrument. In order to check this point Dr. J. T. Littleton, Jr., of that laboratory, has made two redeterminations of the oxyhemoglobin curve on iced material which I sent him. His observations are consistent with my data as they now stand.

The absorption curve for carbon monoxide hemoglobin was obtained from the data of about ten plates made on two solutions

of known oxygen capacity. Only the Nernst lamp was used as a source and the ultra-violet absorption determined out to a little beyond 3100 Ångström units. The depth of the great ultra-violet band was determined on solutions $\frac{1}{10}$ as dense as those otherwise used. It was noticed here too that there were variations in the depth of this band, amounting in a week to a 20 per cent decrease in extinction coefficients for an iced specimen which had been opened several times. The band was brought back to its original depth by again passing illuminating gas through the solution. The height of the peak at 3760 Ångström units decreased likewise very considerably and was as easily restored. Changes in the visible absorption were small if present. The data are derived from freshly gassed solutions.

Concentration of the Solutions.

The concentration of the solutions here used may be calculated from the absorption constants as determined by Hüfner, Cherbuliez, de Saint Martin, Bardachzi, and Butterfield. To do this I calculate from my curve a mean ϵ for the spectrum interval as used by the author in question. The product of this mean ϵ and the author's constant gives a figure for the density of my solution. It happens that the mean ϵ is very nearly equal to the ϵ corresponding to the average transmission for the interval. The data are given in Table I.

If we disregard Butterfield's figures, the concentration would seem to be about 0.85 gm. per liter. As will be seen, however, Butterfield's figures are the correct ones. There is another very direct way of arriving at the concentration of the solutions used, through the determination of the oxygen capacity of the blood used.

Barcroft (23) shows that oxygen combines with hemoglobin in the ratio of one molecule of oxygen to one atom of iron, and further that there is one atom of iron in the hemoglobin molecule. If we accept this proposition, there remains only the question as to what is the percentage of iron in the hemoglobin molecule; what is the molecular weight of hemoglobin? There is very little discrepancy in the figures given by different authors for the iron content of hemoglobin. The accepted figure is about 0.335 per

cent (24). On the basis of this figure the oxygen capacity is 1.34 cc. per gm. of hemoglobin. This figure for oxygen capacity is subject to one correction. It is a maximum figure. Barcroft

TABLE I.

Concentration Data for My Oxyhemoglobin Solutions Based on Regions and Constants of Various Authors.

Author.	Region.	Constant.	Mean ϵ.	Concentration.	Ratio.
Hüfner	α-β	2.070	0.411	0.85	1.54
"	β	1.312	0.633	0.83	
Cherbuliez	α-β	2.038	0.418	0.851	1.577
"	β	1.326	0.659	0.872	
de Saint Martin	α-β	2.153	0.419	0.90	1.556
" " "	β	1.330	0.651	0.865	
Bardachzi	α-β	2.094	0.411	0.86	1.54
"	β	1.334	0.633	0.843	
Butterfield	α-β	1.87	0.409	0.764	1.58
"	β	1.18	0.646	0.762	
"	5460	1.25	0.61	0.763	
"	5770 5790	1.096	0.688	0.754	

(23) has shown that the dissociation curve of hemoglobin (the oxygen saturation curve) has the formula

$$\frac{y}{100} = \frac{Kx^n}{1 + Kx^n}$$

where y is the percentage saturation of the hemoglobin with oxygen, x the oxygen pressure in mm. of mercury, n a number 2.5, and K a constant which varies slightly with normal individuals, averaging about 0.0003.

In measuring oxygen capacity the blood was saturated by spreading it thinly over the inner surface of an open, slowly rotating, conical, separatory funnel. The funnel was so rotated for about 10 minutes before pipetting off the specimen to be examined.

At atmospheric pressure the oxygen pressure is about 180 mm. of mercury. Substituting this figure for x in the formula, y becomes 99.2 per cent. The figure for the cc. of oxygen in 1 gm. of hemoglobin under the above conditions is therefore

$$\frac{99.2}{100} \times 1.34 \text{ cc.} = 1.33 \text{ cc.}$$

Accordingly a 1:201 dilution was made in the various solvents from blood whose oxygen capacity was being determined. The concentration of the solution thus produced when expressed in gm. of oxyhemoglobin per liter was equal to $\frac{10}{201 \times 1.33}$ times the figure for the oxygen capacity of 100 cc. of the whole blood. Extinction coefficients derived from such solutions of known concentration were used to ascertain the concentration of the solutions for which the original absorption curves had been determined. This was accomplished by making use of the fact that the ratio of corresponding extinction coefficients of any two solutions of a substance is equal to the ratio of their concentrations. If the extinction coefficients are known in each case, the concentration of one solution can be deduced from that of the other.

Eight plates were made from four oxyhemoglobin solutions of known concentration. The extinction coefficients at the bottoms of the α- and β-bands as computed from these plates were compared with the figures 0.713 and 0.67 obtained for the corresponding extinction coefficients of the oxyhemoglobin curve. The concentration of this original oxyhemoglobin solution as thus computed at the α- and β-bands was respectively 0.758 and 0.759 gm. per liter. The height of the peak between these two bands as had been computed for Table III was then slightly changed (from 0.408 to 0.406) so as to correspond to a concentration of 0.7585 gm. per liter when similarly referred to the average readings of these eight plates.

If we give these two figures for concentration as derived at the bands an equal weight with the concentration figures of Table I due to Butterfield, we obtain an average of 0.75975 or 0.76 as the gm. per liter of oxyhemoglobin in the solution to which the curve corresponds.

In the case of acid hematin the extinction coefficient of these known solutions at the nearly level interval 5500 to 5580 Ångström units was determined as the average of about a dozen consistent readings to be 0.5185 when computed for a 0.76 gm. solution. The extinction coefficients for the visible absorption of acid hematin were then all multiplied by such a factor as to bring the average extinction coefficient in the region 5500 to 5580 Ångström units to 0.5185.

The values of the extinction coefficients at the tops of the peaks at 3100 Ångström units were in the case of both curves, oxyhemoglobin and acid hematin, such as to correspond to a 0.76 gm. solution. The ultra-violet portion of these two curves was therefore not corrected further for density. The mid-ultra-violet portion of the oxyhemoglobin curve was corrected for oxygen saturation. The extinction coefficients as determined from the carbon monoxide hemoglobin plates, being determined from solutions of known oxygen capacity, were simply reduced by 8.5 per cent so as to correspond to a 0.76 gm. solution.

The intervals chosen by the various authors for the determination of the spectrophotometric constants are too wide. New intervals having a width of 40 Ångström units might be chosen so as to be centrally located. On the basis of the data herein contained such a set of constants for oxyhemoglobin would be as follows:

Oxyhemoglobin.

$A_0 = 1.87$ (5580 to 5620 Ångström units)
$A'_0 = 1.14$ (5390 " 5430 " ")
$A''_0 = 1.068$ (5740 " 5780 " ")

Carbon Monoxide Hemoglobin.

$A_c = 1.343$ (5540 to 5580 Ångström units)
$A'_c = 1.128$ (5360 " 5400 " ")
$A''_c = 1.128$ (5680 " 5720 " ")

Acid Hematin.

$A_h = 1.465$ (5500 to 5580 Ångström units)

Tables II, III, and IV give the absorption data for acid hematin, oxyhemoglobin, and carbon monoxide hemoglobin in an esti-

mated density corresponding to 0.76 gm. to the liter of oxyhemoglobin and in a layer 1 cm. thick.

In Fig. 1 which gives the data of Tables II to IV, extinction coefficients are converted into transmissions, the extinction coefficients being the cologarithms to the base 10 of the transmissions.

TABLE II.

Extinction Coefficients for Acid Hematin Corresponding to 0.76 Gm. of Oxyhemoglobin per Liter.

Wavelength.	Extinction coefficient.	Wavelength	Extinction coefficient.	Wavelength.	Extinction coefficient.	Wavelength.	Extinction coefficient.
Å. u.		Å. u.		Å. u.		Å. u.	
2410	2.06	3340	1.4	4950	0.655	5800	0.349
2440	1.8	3400	1.57	5000	0.625	5840	0.315
2460	1.7	3460	1.74	5040	0.61	5865	0.3
2480	1.65	3500	1.85	5070	0.6	5905	0.28
2540	1.59	3540	1.93	5140	0.59	5960	0.26
2600	1.64	3600	2.0	5200	0.588	6030	0.24
2645	1.7	3680	2.05	5260	0.587	6080	0.23
2670	1.74	3760	2.06	5300	0.583	6140	0.222
2720	1.8	3900	2.0	5320	0.58	6200	0.215
2780	1.81	4000	1.94	5360	0.567	6260	0.2125
2830	1.74	4065	1.9	5400	0.548	6300	0.214
2860	1.64	4100	1.88	5420	0.54	6340	0.22
2870	1.6	4200	1.81	5450	0.53	6400	0.24
2890	1.5	4300	1.73	5480	0.522	6460	0.265
2910	1.4	4390	1.63	5500	0.52	6520	0.29
2930	1.3	4440	1.56	5550	0.5185	6560	0.3
2960	1.19	4500	1.45	5580	0.516	6620	0.305
3000	1.07	4560	1.32	5600	0.5125	6660	0.3
3050	0.98	4600	1.23	5640	0.5025	6700	0.285
3100	0.96	4650	1.125	5660	0.494	6730	0.265
3170	0.99	4710	1.0	5680	0.48	6760	0.24
3210	1.07	4760	0.91	5700	0.459	6800	0.205
3230	1.12	4800	0.84	5720	0.43	6850	0.17
3260	1.19	4860	0.75	5740	0.409	6900	0.14
3300	1.3	4900	0.7	5770	0.375	7000	0.11

TABLE III.

Extinction Coefficients for Oxyhemoglobin, 0.76 Gm. per Liter.

Wave-length.	Extinction coefficient	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.
Å. u.		*Å. u.*		*Å. u.*		*Å. u.*	
2450	2.06	3780	1.25	4750	0.37	5680	0.465
2460	2.0	3800	1.33	4800	0.335	5690	0.5
2480	1.9	3830	1.5	4840	0.31	5700	0.545
2520	1.81	3860	1.75	4920	0.27	5710	0.6
2590	1.77	3890	2.05	4960	0.255	5720	0.655
2660	1.81	3960	3.0	5070	0.233	5730	0.695
2760	1.87	4000	3.7	5120	0.24	5740	0.708
2830	1.81	4040	4.5	5160	0.26	5760	0.713
2870	1.7	4080	5.4	5200	0.295	5770	0.712
2895	1.6	4140	5.7	5240	0.365	5780	0.705
2910	1.52	4200	5.3	5270	0.44	5790	0.665
2930	1.4	4220	4.6	5280	0.462	5800	0.595
2950	1.3	4240	4.0	5300	0.51	5810	0.515
2970	1.2	4260	3.4	5320	0.553	5820	0.465
2985	1.1	4280	2.9	5330	0.58	5830	0.4
3010	1.0	4300	2.5	5340	0.6	5840	0.36
3050	0.9	4330	2.0	5360	0.638	5860	0.265
3100	0.882	4350	1.75	5380	0.66	5880	0.195
3140	0.898	4370	1.5	5400	0.668	5900	0.14
3200	0.98	4400	1.25	5415	0.67	5950	0.087
3230	1.04	4420	1.1	5430	0.667	5960	0.08
3270	1.12	4435	1.0	5440	0.66	5980	0.07
3330	1.21	4450	0.9	5460	0.61	6055	0.05
3400	1.254	4475	0.8	5470	0.58	6140	0.04
3460	1.262	4510	0.7	5480	0.55	6200	0.0355
3520	1.243	4540	0.63	5500	0.495	6300	0.0325
3580	1.155	4570	0.57	5520	0.45	6400	0.03
3600	1.112	4600	0.52	5550	0.42	6500	0.0295
3680	1.065	4650	0.46	5600	0.406	6600	0.0285
3740	1.125	4680	0.43	5650	0.42	6700	0.0275
3760	1.175	4710	0.4	5670	0.445	6800	0.0268

TABLE IV.

Extinction Coefficients for Carbon Monoxide Hemoglobin, 0.76 Gm. per Liter.

Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.
Å. u.		*Å. u.*		*Å. u.*		*Å. u.*	
3000	0.81	4080	4.15	4720	0.347	5660	0.658
3040	0.73	4100	4.9	4760	0.33	5680	0.671
3100	0.7	4120	6.0	4800	0.316	5700	0.675
3160	0.745	4130	6.7	4880	0.299	5720	0.671
3200	0.835	4140	7.4	4980	0.289	5730	0.664
3240	0.96	4150	8.0	5040	0.3	5740	0.645
3280	1.083	4160	8.45	5100	0.34	5760	0.575
3340	1.215	4190	8.7	5150	0.393	5770	0.533
3400	1.3	4220	8.44	5200	0.46	5790	0.445
3460	1.333	4230	8.0	5260	0.55	5820	0.325
3520	1.295	4240	7.3	5300	0.61	5840	0.259
3560	1.215	4250	6.5	5320	0.64	5860	0.213
3590	1.1	4260	5.65	5340	0.66	5900	0.164
3620	0.99	4280	4.25	5360	0.671	5950	0.131
3660	0.89	4300	3.0	5380	0.675	6000	0.111
3700	0.825	4320	1.9	5400	0.673	6040	0.1
3760	0.795	4340	1.4	5420	0.665	6140	0.08
3810	0.82	4360	1.1	5440	0.647	6220	0.07
3840	0.89	4380	0.9	5460	0.6125	6360	0.06
3860	0.985	4400	0.8	5480	0.6	6500	0.054
3880	1.1	4440	0.652	5500	0.581	6600	0.052
3900	1.38	4500	0.535	5550	0.565	6700	0.05
3960	1.9	4560	0.461	5600	0.58	6800	0.049
4000	2.4	4600	0.422	5620	0.6	6900	0.048
4040	3.05	4660	0.379	5640	0.63	7000	0.048

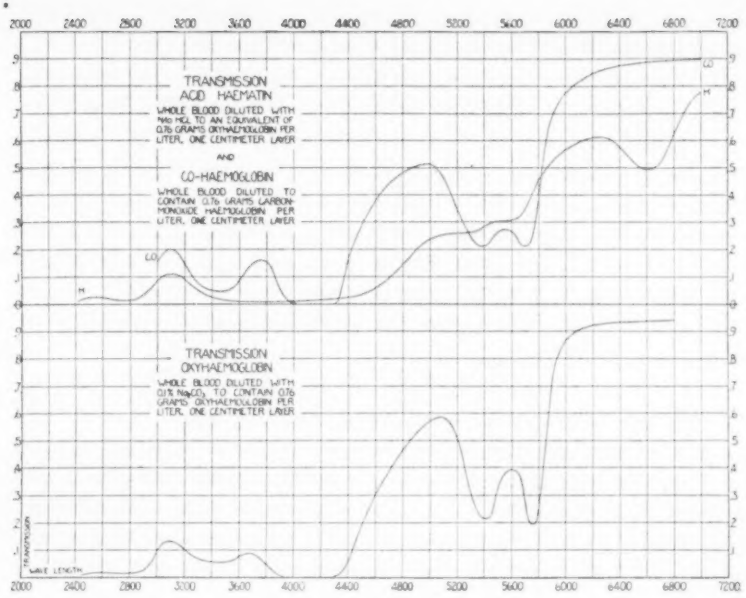

FIG. 1.

DISCUSSION.

Acid hematin is known to have different spectral characteristics under varying conditions as to acid and solvent (4, 5). The curve for material prepared in the manner described is constant and stable, for several days at least. The curve agrees essentially with the spectrogram by Rost, Franz, and Heise (4) of acid hematin prepared in a similar manner and with 1 per cent HCl. The prominent band in the red has its deepest point at about 6620 Ångström units. The difficulty experienced by many (4) in distinguishing photographically the other two bands in the visible is readily understood. They are very shallow. There is a broad general depression extending from 5100 to 5900 Ångström units which is divided by a very slight rise at 5480 Ångström units. In the curve this shows up very distinctly but in a single spectrogram it is discernible only with difficulty, if at all. It first becomes apparent when quantitative absorption measurements are

made. This rise appeared both by the photographic method and with the visual spectrophotometer at Corning.

The great ultra-violet band of acid hematin is broader and shallower than in the case of oxyhemoglobin, the broadening extending it further into the ultra-violet and obliterating the small peak at 3680 Ångström units which appears in the oxyhemoglobin curve. There is beyond the peak at 3100 Ångström units a shallow but distinct band with its center at 2780 Ångström units. Beyond 2400 Ångström units the absorption becomes very great, more rapidly so than in the case of oxyhemoglobin. The further side of this band does not appear within the range of the instrument (2050 Ångström units).

The β-band of oxyhemoglobin is seen to be fainter than the α-band, the difference in depth being, however, slight. As viewed in the ordinary spectroscope there is a subjective difference greater than the real difference. The great ultra-violet band is much deeper, the ratio of the extinction coefficients being about eight. This means that the ultra-violet band can be recognized in eight times as dilute a solution as can the visible bands. A quartz spectrograph is almost necessary.

Oxyhemoglobin has two further bands in the ultra-violet, at 3460 and at 2760 Ångström units separated by a peak at 3100 Ångström units somewhat higher than the corresponding one of acid hematin. The similarity in the ultra-violet of the absorption of the two substances would seem to indicate that it is due in large part to a radical common to both. In fact, this similarity persists, to a less extent, in the visible.

Carbon monoxide hemoglobin has an absorption similar to that of oxyhemoglobin. The differences are interesting; in the visible there is a reduction in the amplitude of the curve, the bands are not so sharp, and the transmission is not so great, particularly at the peak between the α- and β-bands. It is the lowness of this peak which principally accounts for the difference in shade between solutions of the two compounds. The visible bands of carbon monoxide hemoglobin are approximately of the same depth as the β-band of oxyhemoglobin; they are shifted with respect to those of oxyhemoglobin toward the violet; the great ultra-violet band is much deepened and shifted in the contrary direction. In the ultra-violet, the two peaks are higher than in the

case of oxyhemoglobin. The height of the peak at 3760 Ångström units and the depth of the band at 4190 Ångström units decreases very markedly with loss of carbon monoxide from the solution, a change which also holds for oxyhemoglobin.

According to Lewis (25) beyond 3100 Ångström units serum shows a rapidly increasing amount of absorption which reaches a maximum at about 2800 Ångström units, the next minimum being at 2540 Ångström units. The forms of the acid hematin and oxyhemoglobin curves in this region are probably due to the serum present in an approximate dilution of 1:200. In particular the peak at 2540 Ångström units would seem to be due to this. The amount of the extinction due to the admixed serum is only from one-fourth to one-seventh of the whole.

A 1 cm. layer of undiluted blood serum has an absorption which in the red part of the spectrum amounts to an extinction coefficient of about 0.2. This extinction increases uniformly across the visible spectrum to reach a value of about 0.7 in the blue. From the ultra-violet border out to 3200 Ångström units the extinction coefficient of serum is approximately 1. It varies probably with the food taken. I have observed the extremes of 0.8 and 1.7.

In a dilution of 1:200, therefore, serum has an extinction varying from 0.001 to 0.004 in the visible spectrum, being only 0.0015 at its middle point. These figures added to, or subtracted from the extinction coefficients of the hemoglobins do not change them appreciably. The presence of the plasma can therefore be neglected in discussing the visible absorption of these compounds.

The oxyhemoglobin curve as given is approximately that due to a layer of whole blood 0.05 mm. thick. Such a layer is equivalent to a layer of red blood cells in heterogeneous position nine thick, or in rouleau, fifteen thick.

The blood in the superficial capillaries of the skin would therefore be sufficient in quantity to absorb almost completely all the violet and ultra-violet light which might fall upon it. This absorption is particularly intense in the neighborhood of 4200 Ångström units.

Hemotometry.

The banding in the visible of solutions of these three compounds makes it extremely difficult to secure artificial color matches for them. It would be desirable to find an artificial

material whose absorption curve coincided throughout with that of one of them. Under such circumstances the match would appear perfect in any light and to any eye. As the absorption curve of the material to be used as a match deviates more and more from that of the blood preparation, factors are introduced which make it exceedingly difficult to predict from the curves the character which the match will assume.

That a match then exists at all is due to physiological conditions, to the fact that the sum of two colors gives to the eye an impression of a third, a mixed color.

Thus a piece of glass transmitting only green and orange light would in daylight appear to have the same color as a piece of glass transmitting yellow light. But if pure spectral green light were used as an illuminant, the one glass would appear green, the other would be opaque. The two substances may thus appear to have the same color without their absorption curves being at all alike. If their absorption curves are alike, or nearly so, they will appear to have the same color under widely varying conditions, both as to observer and illumination. It is thus essential in order that a color match be satisfactory under varying conditions that the absorption curves of the two substances composing the match be as near alike as possible.

The similarity must be greatest in the region of maximum luminosity, a region whose middle point in the case of ordinary white light is 5560 Ångström units (26). Toward the two ends of the visible spectrum the luminosity is so small that discrepancies here do not materially affect the impression on the eye.

The Miescher hemoglobinometer uses as a match for oxyhemoglobin a gold ruby glass. This glass has a broad absorption band with its center at 5200 Ångström units, which in general balances the two absorption bands of oxyhemoglobin. The transmission of this glass in thickness corresponding to the oxyhemoglobin solution of 0.76 gm. to the liter is given in the upper part of Fig. 2, Curve A. Its absorption only roughly approximates that of oxyhemoglobin. That a match exists at all is, so to speak, accidental, and then only under fixed conditions of illumination.

The upper portion of Fig. 2 contains two other curves, B and C, obtained by multiplying the transmission curves of the Miescher glass and of oxyhemoglobin respectively by the visibility curve

for the Hefner lamp, whose luminosity values are very similar to that of the candle. These two modified curves might be called the physiological transmissions of the two materials. They represent the actual appearance to the eye of each of the two materials when illuminated by the candle. The transmissions of the glass and of the oxyhemoglobin at each point of the spectrum are decreased at each point to correspond to the actual impression which the transmitted light produces on the eye.

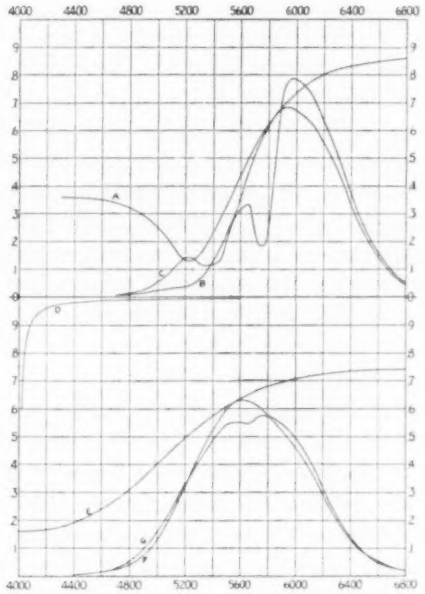

FIG. 2. Curve D is a plot of the equation $xy = -40$, the abscissæ representing time in minutes and the ordinates the percentage short of full color of an acid hematin solution, the squares being each 10 units. For the other curves the ordinates represent transmissions in tenths and the abscissæ wave-lengths in Ångström units. A is the Miescher glass in a thickness corresponding to the oxyhemoglobin of Fig. 1. B is the same multiplied by the visibility of the Hefner lamp. C is the oxyhemoglobin curve multiplied in the same manner. E is the transmission of 1 mm. of high transmission yellow colorimeter glass. F is the same multiplied by the visibility of white light. G is a corresponding acid hematin transmission so multiplied. It is assumed that there is a sufficient intensity of illumination to make applicable the visibility values used.

The amount of agreement or disagreement between these two physiological transmission curves gives an interpretation of the quality of the match. The area under each curve represents the quantity of light which each material appears to transmit. The two areas are equal, the light fields being presumed to balance.

There does not seem to be any way to improve on this match for oxyhemoglobin. There is no red glass that falls to zero transmission near 5800 Ångström units. They all come down to zero much further in the red, presenting a distinctly different hue. It is possible by using an illuminant that is entirely red to confine the problem to the level portion of the high transmission red part of the oxyhemoglobin curve. Then the shades match exactly, but the transmission of the oxyhemoglobin is there so high and the sensitivity of the eye in the middle and far red so low that it is impossible to distinguish any but gross differences in color. The same situation exists in the case of carbon monoxide hemoglobin, except that its relatively low peak between the two visible bands makes it possible to seek a glass whose single band will coincide with the position of this peak. Unfortunately glasses having high transmissions in the blue and red have their central bands too far over in the green. In the case of acid hematin the situation is better.

I have examined at the laboratory of the Corning Glass Works a collection of about 1,000 colored glasses, representing the possibility of a million pairs. There is no glass or combination of glasses giving an absorption whose curve runs closely parallel to that of acid hematin. The "high transmission yellow" semaphore glass made by the Corning Glass Works, however, has an absorption curve which runs smoothly as a mean through the acid hematin curve. It has not been possible to combine this with any other glass so as to produce a combination having a curve more closely approximating that of the acid hematin than does the yellow glass alone.

This glass matches acid hematin best when to its specific absorption is added the flat absorption due to reflection losses at its surface. In colorimetric comparison, therefore, the yellow glass is not to be balanced by a corresponding piece of clear glass, but is to be used alone on one side of the color balance. The hematin solution is, however, to be balanced for reflection

losses at its surface by clear water on the colored glass side of the balance.

This glass in thickness of about 1 mm. is a very close match for acid hematin and the discrepancy between its curve and that of the acid hematin is sufficiently small so that the match does not perceptibly vary with individuals. In this thickness the depth of color is about an optimum for judging differences of color depth.

Curve E, Fig. 2, gives the transmission of a piece of this glass 1 mm. thick. Curves F and G are the physiological transmissions of this glass and of a corresponding acid hematin solution respectively. They are obtained by multiplying the transmissions of the two materials by the luminosity curve for white light. It will be seen that they fall more nearly together than do the corresponding Curves B and C for the Miescher hemoglobinometer.

In order that the glass shall match a fixed density acid hematin solution the illumination must be constant. It may be either the light of a northern sky or that given by a nitrogen-filled tungsten lamp filtered through "daylite" Corning glass, the two illuminants being interchangeable. The match is sufficiently good so that further improvement is not a matter of importance, there being a limit to the refinement with which any colorimetric estimation may be made. The use of a fixed thickness of colored material as a standard has a distinct advantage over the use of a wedge. It is impossible to secure an artificial color match which shall run true through a range of thicknesses, though some may do so approximately.

Clinical Estimation of Oxyhemoglobin.

There is no method of estimating hemoglobin which is superior to the oxygen capacity method of Van Slyke (21). For clinical purposes, however, some colorimetric method for the estimation of hemoglobin is essential. It therefore becomes a question of devising a color standard which shall be permanent, which to different individuals shall have the same apparent hemoglobin value, and which can be easily supplied as a uniform product. A stock of "high transmission yellow" glass having the properties set forth in the previous section has been set aside for this pur-

pose.[2] Its hemoglobin value has been carefully estimated. It is to be used in thicknesses of 1 mm. in connection with some colorimeter of the Duboscq type. When used with the Duboscq colorimeter it is placed in the light path of one of the cells (at the top of the plunger) and the corresponding cell or cup is filled with water. In the other cup are placed 5 cc. of 0.1 N HCl (1 per cent C.P. HCl) and to the cup are added 20 c.mm. of blood obtained from a puncture wound with a capillary pipette.[3] If the two light fields are now balanced by lowering the plunger in the hematin cup the hemoglobin reading in gm. per 100 cc. of whole blood is represented by the quantity $\frac{0.38d}{t}$ where d is the dilution (in this case 251), t the thickness of the balancing solution in mm., the colorimeter reading, and 0.38 a number empirically determined for a 1 mm. glass.[4] This expression has to be modified

[2] The Arthur H. Thomas Company, Philadelphia, is prepared to supply this glass cut, ground, and polished to thickness, the thickness being engraved on each piece. They will also supply the necessary pipettes, and the "daylite" unit for use at night consisting of an 8" roundel of "daylite" glass and an appropriate shade. The measured glass discs will also be provided as a part of the regular equipment of the new Bausch and Lomb colorimeter of the Duboscq type.

[3] The colorimeter described by Bock, J. C., and Benedict, S. R., (*J. Biol. Chem.*, 1918, xxxv, 227) is well adapted for this purpose. The colorimeter cup is small enough to be almost filled by 5 cc. of solution, thus permitting readings to be made on bloods running as low as 20 per cent of the normal without its being necessary to increase the density of the hematin solution by using a larger amount of blood. For the ordinary Duboscq cup the 20 c.mm. of blood suffice for hemoglobin readings down to 50 per cent of the normal.

[4] Since the thickness of the glass can seldom be exactly 1 mm., there is a further factor multiplying this formula. If h be the thickness of the glass, then for values of h sufficiently near unity it is permissible for this factor to be h itself. As a matter of fact it is slightly less than h. For such an approximation h should not vary from unity by more than 0.05 mm. The actual value of the factor is somewhat involved. If $\phi(\lambda)$, λ being wavelength, represents the values of the extinction coefficients for a 1 mm. glass alone (not including surface loss), then for the glass and surface together $\epsilon = \phi(\lambda) + 0.041$ and the transmission is $T = 10^{-\phi(\lambda) - 0.041}$ (Curve E, Fig. 2). The total energy transmitted by the glass in the visible region is the area under this curve, or

$$E = \int_{\lambda = 4000}^{\lambda = 7000} 10^{-\phi(\lambda) - 0.041}\, d\lambda$$

because of the fact that the hematin does not reach its full density for some time (as stated above). If x is the time in minutes after making the dilution from the pipette, then the reading is too small by a percentage equal to $\frac{40}{x}$, and the real reading is

$$\frac{38d}{t\left(100 - \frac{40}{x}\right)}$$

It will be seen that when x is very large this becomes equivalent to the first formula given. When x is equal to 10 (at the end of

But, as mentioned above, we are concerned rather with the physiological effect of this energy than with the energy itself. If the visibility of white light is represented by the function $\theta\ (\lambda)$, then this physiological energy impression or the luminosity of the illuminated glass is

$$P = \int_{\lambda = 4000}^{\lambda = 7000} \theta\ (\lambda)\ 10^{-\phi\ (\lambda) - 0.041}\ d\lambda$$

The thickness of the glass being h instead of unity, the new physiological transmission integral is

$$P_h = \int_{\lambda = 4000}^{\lambda = 7000} \theta\ (\lambda)\ 10^{-h\,\phi\ (\lambda) - 0.041}\ d\lambda$$

If $\psi\ (\lambda)$ represents the extinction coefficient of a solution of acid hematin matching the glass given by $\phi\ (\lambda)$, then a condition for the match is

$$\int_{\lambda = 4000}^{\lambda = 7000} \theta\ (\lambda)\ 10^{-\psi\ (\lambda)}\ d\lambda = \int_{\lambda = 4000}^{\lambda = 7000} \theta\ (\lambda)\ 10^{-\phi\ (\lambda) - 0.041}\ d\lambda$$

and this condition holding, then

$$\int_{\lambda = 4000}^{\lambda = 7000} \theta\ (\lambda)\ 10^{-h\,\psi\ (\lambda)}\ d\lambda \neq \int_{\lambda = 4000}^{\lambda = 7000} \theta\ (\lambda)\ 10^{-h\,\phi\ (\lambda) - 0.041}\ d\lambda$$

but the two integrals are nearly equal when h is nearly unity. No matter how nearly $\phi\ (\lambda)$ may equal $\psi\ (\lambda)$ these two latter integrals cannot be alike because of the factor $10^{-0.041}$ in one of them. If conditions are such that the surface factor $10^{-0.041}$ is absent then, since the assumption is that the functions $\phi\ (\lambda)$ and $\psi\ (\lambda)$ are not identical, it follows that the integrals also are not identical. They can be made equal for one and only one value of h, a value here chosen as unity. This inequality constitutes sufficient proof of the proposition that a wedge of glass can never exactly balance a varying solution density. The accompanying inequalities in shade constitute the most important difference.

10 minutes), the hematin has reached to within 4 per cent in 20 minutes to within 2 per cent, and in 40 minutes to within 1 per cent of its final value. For mental calculations the figure given by the simple formula $\frac{0.38d}{t}$ may therefore be simply increased by these percentages.

Table V gives some of the readings by which the calibration of the glass was made and indicates what latitude may be expected in such observations. The table gives results with the artificial illumination and with ordinary daylight. Each column represents ten settings made by the individual whose initials stand at the head of the column. The individuals making the settings were not aware of the readings until they had finished the experiment. It is impossible to expect any single setting to come very close to the mean. This is true of any sort of colorimetric setting though perhaps it is not generally recognized.

A formula giving the oxygen capacity of 100 cc. of blood is obtained by introducing the factor 1.34 into the above formula.

$$\frac{0.38d}{t} \times 1.34 = \frac{0.51d}{t}$$

Haldane and Smith (27) adopt 18.5 cc. oxygen capacity as a normal. The resultant hemoglobin percentage scale is known by his name. On his scale the formula for per cent hemoglobin is $\frac{2.75d}{t}$ per cent.

Tables VI and VII give in convenient form the figures into which t, the thickness of the balancing solution in mm., is to be divided in order to obtain the concentration of hemoglobin in gm. per 100 cc. of whole blood and in per cent Haldane. The dilution is taken as 251. To each of certain possible thicknesses of colored glasses there corresponds a column of figures corrected for the time factor in the formation of acid hematin.

TABLE V.

Colorimeter Readings Balancing a Certain Hematin Solution against Yellow Glass.

	H. S. N.	M. G. P.	H. S. N.	M. G. P.	H. S. N.	M. G. P.	H. S. N.	M. G. P.	H. S. N.	M. G. P.	Average
Illumination with "daylite" unit.											
	4.05	3.9	4.05	4.2	4.0	3.8	4.0	4.05	4.0	4.0	48.60
	4.0	3.8	4.05	4.0	4.0	3.9	4.1	4.15	4.05	3.8	47.60
	4.0	4.15	4.1	4.15	4.1	4.1	4.0	4.15	3.95	3.9	48.35
	4.0	4.05	4.0	4.0	4.3	4.1	4.05	4.1	4.2	3.8	47.65
	4.05	4.0	4.25	3.8	3.9	4.2	4.15	4.1	4.2	4.2	48.05
	4.3	3.85	4.4	3.8	4.15	3.9	3.85	4.3	4.1	4.15	48.25
	4.05	4.05	4.1	4.1	4.4	4.1	3.95	4.1	4 0	4.1	47.40
	4.0	4.05	4.35	3.9	4.0	4.05	4.25	3.8	4.15	4.1	47.60
	4.25	4.15	4.05	3.9	4.0	4.15	3.95	3.95	4.15	4.1	47.90
	4.1	3.8	4.0	4.0	4.2	4.15	4.1	3.9	4.1	3.8	46.95
	40.80	39.8	41.35	39.85	41.05	40.45	40.40	40.60	40.90	39.95	478.35
Zero correction.	7.8	7.8	7.0	7.8	7.0	7.8	7.0	7.0	7.0	7.0	
	48.60	47.60	48.35	47.65	48.05	48.25	47.40	47.60	47.90	46.95	
Illumination with light of northern sky.											
	4.15	4.0	4.05	4.05	4.0	4.0	3.95	3.95	4.05	3.8	48.05
	4.05	3.9	4.0	3.8	3.9	4.2	3.95	4.0	4.0	4.0	47.50
	4.15	3.9	4.05	4.05	4.0	4.1	3.9	4.15	4.1	3.8	47.55
	4.2	3.95	3.9	4.0	4.0	4.0	3.9	4.0	4.1	4.05	47.00
	4.15	4.2	4.05	3.95	3.95	4.0	3.95	3.9	4.05	4.15	47.80
	4.0	4.2	4.0	3.95	4.0	4.0	3.9	3.85	4.1	4.15	48.40
	4.05	4.1	4.0	4.0	3.85	4.0	4.15	4.1	3.85	4.0	47.85
	4.0	4.05	4.05	4.1	4.05	4.0	4.05	4.0	4.1	4.15	48.15
	4.1	4.0	4.25	4.1	3.95	4.0	4.05	4.1	4.05	4.2	48.40
	4.2	4.2	4.2	4.0	4.1	4.1	4.05	4.1	4.0	4.2	48.50
	41.05	40.50	40.55	40.00	39.80	40.40	39.85	40.15	40.40	40.50	479.20
Zero correction.	7.0	7.0	7.0	7.0	8.0	8.0	8.0	8.0	8.0	8.0	
	48.05	47.50	47.55	47.00	47.80	48.40	47.85	48.15	48.40	48.50	

Average with "daylite" unit		478.35
" " light from northern sky		479.20
Final average	200	957.55
		4.7877

Oxygen capacity of blood used..... = 20.6 cc.
Dilution..... = 201
Colorimeter factor..... = 0.369
Thickness of colored glass..... = 0.97 mm.
Colorimeter factor corrected to 1 mm. glass = 0.38

TABLE VI.

To obtain gm. of hemoglobin per 100 cc. of whole blood, divide the colorimeter reading into the figure of the appropriate square, the choice of the square depending upon the thickness of the glass used and the length of time since dilution of the blood was made.

Time since dilution.	Thickness of colored glass in mm.										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
min.											
10	94.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.4	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final.....	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

TABLE VII.

To obtain the per cent hemoglobin, Haldane scale, divide the colorimeter reading into the figure of the appropriate square, the choice of the square depending upon the thickness of the glass used and the length of time since dilution of the blood was made.

Time since dilution.	Thickness of colored glass in mm.										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
min.											
10	683	690	697	704	712	719	726	733	740	748	755
15	673	680	687	694	702	709	716	723	730	737	744
20	669	676	683	690	697	704	711	718	725	732	739
30	664	671	678	685	692	699	706	713	720	727	734
40	662	669	676	683	690	697	704	711	718	725	732
Final.....	655	662	669	676	683	690	697	704	711	717	724

CONCLUSION.

The transmissions of acid hematin, oxyhemoglobin, and carbon monoxide hemoglobin for the ultra-violet and visible spectra have been determined to within limits of error, which I believe do not exceed 4 and 2 per cent respectively. The quantitative data for the oxygen capacity of hemoglobin has been shown to agree essentially with certain spectrophotometric data and the two have been brought into correlation with each other and with the complete absorption curves of the three compounds. The optical

conditions underlying colorimetric hemotometry have been briefly discussed. A hemoglobinometer with a colored glass standard has been described.

I am indebted to many for the interest which they have shown in the experimental difficulties which have presented themselves. More particularly I wish to thank Drs. Littleton and Gage of Corning and Dr. Karrer of the United Gas Improvement Company for the facilities which they have extended to me.

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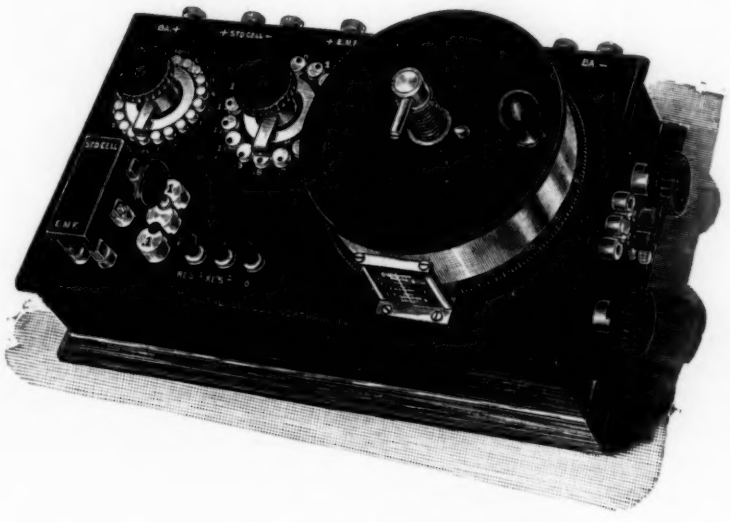

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